◆ *COTUTELLE ENTRE* ◆

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◆ *ET* ◆

# L'UNIVERSITÉ LIBRE DE BRUXELLES

***FACULTÉ DES SCIENCES***

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## **THÈSE** présentée par :

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# Coevolution of sociality and ageing in animal societies

***Coévolution de la socialité et du vieillissement dans les sociétés animales***

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Lors des trophallaxies, ces échanges de nourritures de bouche à bouche entre deux individus observés chez les insectes sociaux, les fourmis échangent en réalité bien plus que des nutriments (molécules de reconnaissance, protection immunitaire, hormones…). Il en va de même, pour nos repas partagés entre doctorants qui ont été l'occasion de débats toujours menés avec passion quelqu'en soit le sujet (la bienséance m'oblige à ne pas dévoiler tous les thèmes abordés…) ! Heureusement, nous avons su nous détacher du modèle fourmi et n'échangeons pas tous nos repas par trophallaxies… En revanche, imitant les Fourmis qui ont inventé l'agriculuture bien avant les

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Ce travail, c'est un peu le vôtre aussi.

# Résumé en français

Le résumé ci-dessous, reprend les grandes parties de cette thèse : **A)** le contexte historique et scientifique de l'étude du vieillissement, notamment les théories évolutives du vieillissement, les mécanismes molécualires associés et liens avec la socialité à l'échelle des espèces et des individus (pages v-xii) ; **B)** les objectifs, l'intérêt et l'organisation de ce travail de thèse (pages xii-xiv); **C)** le résumé des résultats principaux (pages xiv-xvi); et **D)** des voies que pourraient emprunter de futures recherches pour mieux comprendre ces liens entre vieillissement et socialité (page xvi). Bien qu'encore parfois un peu technique, le but de ce résumé et d'essayer de communiquer en français le contexte scientifique et les résultats principaux de ma thèse, même pour un public non averti. L'ensemble des points abordés dans ce résumé peuvent être trouvés en plus amples détails dans le corps principal du manuscrit, ainsi que les références bibliographiques que je n'ai pas reportées ici. Bonne lecture.

## A) Contexte historique et scientifique

### 1. La compréhension du vieillissement au cours des âges

Pourquoi et de quoi meure-t-on lorsque la mort survient de façon naturelle ? Voilà, peut-être une des plus vieilles questions que l'humanité se pose. Dans l'antiquité, la mort, la vieillesse et les maladies associées sont inévitables, issues de la volonté de divinités, souvent infligées aux Hommes pour punir une faute (mythes antiques de Prométhée, Gilgamesh, Adam et Ève). En opposition à ce point de vu fataliste, au VIIIème siècle, l'alchimiste Al Jabir écrit de nombreux textes qui diffusent dans le monde Arabe et en Europe des préceptes taoïstes pour atteindre des longévités extrêmes. Plus tard, le philosophe et scientifique anglais Roger Bacon (1214-1294) et l'écrivain italien Luigi Cornaro (~1460-1566) contribueront fortement à répandre en Europe l'idée de la possibilité d'allonger considérablement la durée de vie humaine en adoptant un mode de vie sains, sans excès. Au cours du XVIIIème siècle, les progrès de la médecine et de la science en général amènent les partisans de l'extension de la longévité à se tourner vers une étude plus scientifique des causes des maladie et de la vieillesse. Depuis, l'humanité a accumulé de nombreuses connaissances et progressé technologiquement. Néanmoins, le vieillissement, qui se définit en biologie comme une perte progressive de la capacité d'un organisme à maintenir ses fonctions vitales au cours du temps, n'a pas encore révélé tous ses secrets. À la lumière de la théorie de l'évolution des espèces par la sélection naturelle, l'existence même du vieillissement biologique semble paradoxale. En effet, d'après cette théorie, les êtres vivants sont sélectionnés sur leur capacité à maximiser la transmission de leurs caractéristiques génétiques aux génération suivantes. Ils

doivent donc survivre suffisamment longtemps pour pouvoir se reproduire et se reproduire avec le meilleur taux de succès possible. Or, le vieillissement agit négativement à la fois sur la survie (p. ex. perte de force et d'endurance musculaire, diminution des défenses immunitaires) et sur les capacités à se reproduire (augmentation de l'infertilité avec l'âge). Dans ce contexte, on s'attendrait à ce que la moindre mutation qui permettrait d'empêcher le vieillissement soit fortement sélectionnée et se retrouve à l'heure actuelle chez la majorité des êtres vivants. Or, à part des méduses du genre *Turritopsis* capables d'inverser leur cycle de développement et ainsi de « rajeunir », très peu d'espèces semblent capables de s'affranchir des mécanismes délétères du vieillissement. Il reste donc à expliquer pourquoi le vieillissement et la mort qui en découle n'ont pas été contre-sélectionnés de manière plus efficace chez davantage d'espèce.

### 1.1. Théories évolutives du vieillissement.

Pour qu'un gène soit sélectionné et perdure au sein d'une espèce, il doit procurer un avantage à l'individu qui l'exprime et pouvoir se transmettre à la descendance. Les gènes qui procurent un avantage après la période reproductive de la vie, ne permettent pas d'avoir plus de descendants, donc ces gènes ne sont pas spécifiquement sélectionnés. Pour la même raison, un gène qui procurerait des désavantages dans la période post-reproductive de l'individu ne peut être efficacement éliminés. Par conséquent, les théories suivantes ont pour point commun d'expliquer comment les gènes impliqués dans la sénescence peuvent passer cette fenêtre temporelle de la sélection (entre la naissance et la fin de la vie reproductive). Une des premières théories modernes du maintien de la sénescence dans l'histoire évolutive du vivant est celle dite d'*accumulation des mutations*, développée par Medawar et Haldane. Celle-ci postule que les animaux ont de grandes chances de mourir à cause de facteurs externes présents dans leur environnement, tels que la prédation ou les pathogènes, bien avant de mourir du vieillissement de leur organisme. Les mécanismes permettant d'échapper à ces facteurs externes serait donc davantage sélectionnés que ceux en faveur de mécanismes de maintenance de l'organisme sur le long terme. Les mutations de l'ADN et les dommages sur les autres molécules du vivant (lipides, protéines…) s'accumulent donc au cours de la vie de l'individu sans être suffisamment réparés et finissent par causer le dépérissement de l'organisme. Dans cette théorie, la sénescence apparaît comme un produit secondaire du fonctionnement de l'organisme. George C. Williams propose, lui, une autre théorie appelée *pléiotropie antagoniste*. La pléiotropie est la possibilité pour un gène d'avoir plusieurs fonctions, notamment au cours de la vie de l'individu. Un gène pléiotrope a des effets bénéfiques (meilleure reproduction, meilleure survie…) en début de vie, ce qui explique sa sélection et transmission à la descendance, puis des effets négatifs apparaissent après la vie reproductive de l'individu et le gène ne peut alors plus être éliminé par la sélection naturelle. Par exemple, les enzymes Nox et Duox

participent aux défenses immunitaires, mais sont aussi mises en cause dans l'apparition de maladie chronique en fin de vie, comme l'hypertension, l'athérosclérose, ou encore Alzheimer. Kirkwood ajoute à ces deux précédentes théories la notion de *compromis énergétique.* Cette hypothèse part du constat que les ressources disponibles dans l'environnement sont limitées. Ce qui induit une compétition pour les ressources énergétiques entre les différentes fonctions de l'organisme. Par exemple, lors de la saison de reproduction davantage d'énergie est allouée à cette fonction plutôt qu'à des mécanismes de réparation de l'organisme. Ces hypothèses, non mutuellement exclusives, définissent le cadre évolutif théorique de la biologie du vieillissement. Ce cadre théorique permet d'imaginer comment la sénescence peut échapper à la sélection naturelle mais ne dit pas par quels processus biologiques vieillissent les organismes.

### 1.2. Les mécanismes moléculaires du vieillissement

La première hypothèse à proposer un mécanisme cellulaire précis a été théorisée par Denham Harman dans les années 1950 sous le nom de *théorie radicalaire du vieillissement*. Un radical désigne en chimie un atome ou une molécule qui a une forte probabilité d'interagir avec d'autres molécules en leur enlevant des électrons. Le fait d'enlever un ou plusieurs électrons à une molécule s'appelle en chimie une oxydation. Cette oxydation modifie profondément les propriétés de la molécule « endommagée » par le radical. Dans les cellules animales, l'oxygène est utilisé pour fournir de l'énergie aux cellules. Cependant, une partie de l'oxygène échappe à ce processus de synthèse d'énergie à et va réagir avec d'autres molécules et former des radicaux qui à leur tour vont oxyder les biomolécules (ADN, protéines, lipides). Certaines molécules sont capables de bloquer ces réactions dommageables d'oxydation, elles constituent ce qu'on appelle la « barrière antioxydante » de l'organisme. Lorsque cette barrière est dépassée par la quantité de molécules oxydantes, c'est là que des dommages apparaissent et on parle alors de stress oxydant. Ce stress oxydant peut mener à des dysfonctionnements importants si trop de molécules sont endommagées : mutations de l'ADN, protéines qui perdent leur efficacité…

Le stress oxydant fait un bon coupable du vieillissement : l'activité du métabolisme génèrent un plus grand besoin d'oxygène, donc plus de possibilité de former des radicaux et de créer du stress oxydant qui endommage les molécules biologiques. L'explication est simple et explique la tendance générale des animaux avec un métabolisme élevé (souris) à vieillir plus vite que ceux avec un métabolisme plus lent (éléphants, tortues). Néanmoins, plusieurs études montrent que les radicaux, loin d'être des molécules uniquement délétères s'avèrent parfois essentiels dans la transmission de messages chimiques au sein des cellules. Ceci montre que les molécules oxydantes et anti-oxydantes font en fait partie d'un équilibre dont les deux composantes sont essentielles au bon fonctionnement de

l'organisme. Par ailleurs, bien que plusieurs études montrent une association négative entre stress oxydant et longévité, ce n'est pas le cas de toutes les études. Par exemple, des essais cliniques avec une prise d'antioxydants n'ont pas pu démontrer une augmentation la longévité. Chez l'abeille domestique comme chez la fourmi noire des jardins des gènes codant pour des molécules antioxydantes, ont été retrouvés moins fortement exprimés chez les reines qui vivent pourtant plus longtemps que les ouvrières. Le stress oxydant, bien que lié aux mécanismes de la sénescence ne semble donc pas en expliquer toute la complexité.

Nos cellules se divisent, une cellule mère se scinde en deux et donne donc deux cellules filles. Ce phénomène appelé division cellulaire permet la croissance et le remplacement des cellules tout au long de la vie d'un individu. Au cours de cette division, chaque cellule fille reçoit la moitié du patrimoine génétique de la cellule mère. Mais pour fonctionner correctement, nos cellules ont besoin de la totalité du matériel génétique. Les cellules filles recopient donc à l'exemplaire d'ADN qu'elles ont reçu. Cette étape s'appelle la réplication de l'ADN. Malheureusement, elle n'est pas parfaite, des modifications peuvent avoir lieu. Notamment, l'extrémité des chromosomes n'est jamais recopiée entièrement. Donc à chaque division cellulaire, ces extrémités raccourcissent. Afin d'empêcher que des séquences d'ADN codant pour des gènes vitaux ne soient endommagés par ce raccourcissement, il existe des séquences d'ADN nommées télomères qui sont justement placées aux extrémités des chromosomes. Ces télomères se font donc raccourcir à la place des autres séquences du génome. Néanmoins, au bout d'un certains nombres de divisions les télomères ont trop raccourcis et ne peuvent plus assurer leur rôle protecteur. Lorsque ce stade est atteint, la cellule déclenche alors des voies de signalisations qui vont mener à sa mort programmée que l'on nomme l'apoptose. Par exemple chez l'humain, ce seuil, appelé limite de Hayflick, est d'environ 50 divisions avant que la cellule ne meure. Plus les télomères sont courts ou plus ils raccourcissent rapidement, plus une cellule mourra rapidement entraînant une diminution des cellules fonctionnelles dans l'organisme et donc des dysfonctionnements potentiels. La taille des télomères à la conception apparaît contrôlée en partie par des facteurs génétique et héritable. Par exemple, une étude menée auprès de jumeaux du même sexe a montré que non seulement la taille des télomères des globules blancs humains était héritable (à 64% avec 22% d'influence partagée avec l'environnement), mais aussi leur vitesse d'érosion (à 72% sans influence partagée). Les télomères reflètent aussi les compromis énergétiques que doit opérer un individu au cours de son histoire de vie. Par exemple, il a été démontré que les individus contraints à se développer plus rapidement, et donc à négliger la maintenance de l'organisme au profit de la croissance, présentaient des télomères plus courts. Par ailleurs, la composition moléculaire-même des télomères en fait une cible privilégiée des molécules oxydatives, expliquant le raccourcissement des télomères en conditions de stress oxydant.

Les télomères apparaissent donc comme un élément de choix pour étudier le vieillissement puisqu'ils intègrent à la fois la composante génétique, l'environnement et qu'ils reflètent les compromis énergétiques au cours de la vie. Chez les oiseaux et les mammifères, les preuves s'accumulent montrant que la vitesse d'érosion des télomères représente un excellent prédicteur de la longévité d'un individu, bien meilleur que l'âge chronologique. La question se pose alors de savoir si rallonger les télomères permettrait d'augmenter l'espérance de vie. Pour tester cette hypothèses plusieurs expériences ont été tentées en utilisant la télomérase, qui est une enzyme capable d'allonger l'extrémité manquante après la réplication. L'activation de la télomérase via le TA-65, une molécule extraite de la racine d'*Astragalus membranaceus*, a augmenté la durée de vie en bonne santé chez des souris adultes en laboratoire et a amélioré plusieurs indicateurs de bonne santé chez des femmes volontaires. Il faut cependant noter que l'inhibition de la télomérase dans les cellules est un mécanisme prévenant une division infinie et incontrôlée des cellules et donc l'apparition de cancers. Ces données hétérogènes montrent donc que l'extension de la longévité en ralentissant l'érosion des télomères demande davantage d'études pour mieux cerner les processus cellulaires et ainsi prévenir l'apparition d'éventuels effets indésirables. Pour les télomères aussi, la particularité des insectes sociaux et notamment des fourmis peut être soulignée. En effet, contrairement à la grande majorité des espèces de Vertébrés, ni l'activité de la télomérase, ni la longueur des télomères ne reflètent la différence de longévité entre les reines et les ouvrières de la fourmi noire des jardins. Cependant très peu d'espèces d'insectes ont été étudiées de ce point de vue et c'est d'ailleurs à cette tâche que s'est attelé le Chapitre 3 de ma thèse.

Nous avons vu ci-dessus des mécanismes de vieillissement liés aux dommages sur les macromolécules du vivant (p. ex. protéines, lipides, ADN) au cours de la vie d'un individu, avec une emphase particulière sur les télomères qui semblent un mécanisme très conservé et au pouvoir explicatif puissant, au moins chez les mammifères et les oiseaux. Mais d'autres mécanismes liés à l'activité du métabolisme ont été étudiées plus récemment et semblent prometteurs. De nombreuses études, ont montré que la restriction calorique était plus efficace que la médication pour augmenter la longévité en bonne santé. La restriction calorique ralentit le vieillissement chez les rongeurs, améliore la mémoire chez les humains âgés, retarde la survenue des maladies et de la mort chez les macaques rhésus. Ces bénéfices semblent probablement régulés par au moins deux voies de signalisation interconnectées : la voie dites des sirtuines (des protéines qui interagissent avec l'ADN) et la voie mTOR (mechanistic target of rapamycin) Ces deux voies jouent le rôle de capteurs de l'état énergétique de la cellule. Selon si beaucoup d'énergie (= ressources alimentaires) est disponible alors ces voies vont, par exemple favoriser la croissance ou la reproduction. À l'inverse, s'il y a peu d'énergie disponible, alors ces voies vont plutôt inhiber l'activité du métabolisme et favoriser des mécanismes d'économie d'énergie, de réparation de

l'organisme, etc. À ce jour, les seules expériences ayant accru de façon claire la longévité dans plusieurs taxa impliquaient la restriction calorique.

## 2. Rôle du contexte social dans le vieillissement

### 2.1. Interconnections entre la socialité et la longévité

Bien que les mécanismes du vieillissement détaillés ci-dessus soient dans l'ensemble communs entre les différentes espèces animales, on observe des variations importantes entre espèces mais aussi entre individus au sein de la même espèce. Ceci met en évidence l'existence de facteurs capables de moduler les mécanismes du vieillissement. Parmi ces facteurs, la vie en groupe joue un rôle important. Les espèces animales montrent des organisations sociales variées qui peuvent être regroupées selon leur degré de complexité. Tout d'abord, les espèces *solitaires* dont les contacts entre individus n'ont lieu qu'au moment de la reproduction, sans soin aux jeunes (p. ex. abeilles solitaires). Puis, les espèces *grégaires* dont les regroupements ne sont dépendants que des conditions environnementales (insectes nocturnes autour d'un lampadaire par exemple). À partir des espèces dites *sub-sociales*, des soins aux jeunes sont observés, même si parfois rudimentaires (p. ex. boulette du scarabée bousier). Les espèces *coloniales* forment de grandes colonies avec une zone d'élevage commune des jeunes mais les parents s'occupent uniquement de leur descendance. Au contraire des espèces *communales* où plusieurs adultes coopèrent dans l'élevage de jeunes même si ce ne sont pas les leurs. Enfin, le degré de socialité le plus élevé est l'*eusocialité*. D'après Wilson et Hölldobler, trois critères doivent être remplis pour qu'une espèce soit qualifiée d'eusociale : le chevauchement d'au moins deux générations, la coopération dans le soin aux jeunes et la division du travail. Cette division du travail signifie que les individus de ces espèces eusociales se spécialisent dans la réalisation d'une tâche au sein de leur colonie. Par exemple, les ouvrières fourrageuses chez els fourmis sont celles qui vont chercher la nourriture à l'extérieur et aucune autre caste d'ouvrières ne sort de la colonie. La division du travail s'applique aussi à la reproduction, puisque seule la reine (ou les reines chez certaines espèces) est fécondée et peut donc se reproduire de façon sexuée. Ce critère de division du travail, notamment en ce qui concerne la reproduction, exclue l'humain de l'eusocialité. Chez les mammifères, seules deux espèces de rats-taupes sont considérées comme strictement eusociales le rat-taupe nu (*Heterocephalus glaber*) et le rat-taupe de Dammara (*Cryptomys damarensis*). Chez les invertébrés quelques espèces de crevettes sont aussi décrites comme eusociales, mais le groupe qui comprend le plus d'espèces eusociales est celui des insectes sociaux : termites, fourmis et certaines espèces d'abeilles et de guêpes.

La vie en groupe apporte des bénéfices immédiats quant à la survie des individus des espèces qui adoptent ce mode de vie : se défendre contre les prédateurs, trouver et garder la nourriture, défendre

son territoire… De cette façon, les individus qui vivent en groupe ont plus de chances de vivres plus longtemps. Il devient alors intéressant de sélectionner des mécanismes de réparation de l'organisme sur le long terme, puisque l'individu a désormais des chances d'atteindre un âge où de tels mécanismes pourraient s'avérer utile pour lutter contre une accumulation de dommages au cours de sa vie. De plus, lorsque l'on vit en groupe, on peut continuer à protéger nos descendants. Donc leur survie dépend aussi de notre propre survie, ce qui favorise une fois encore la sélection de gènes codant pour des mécanismes de longévité, afin de vivre le plus longtemps possible en bonne santé et s'assurer que nos descendants soient en bonne santé et aient à leur tour le plus de descendants possibles, ce qui assure la dissémination de notre patrimoine génétique de génération en génération.

La comparaison d'espèce eusociales avec leurs équivalents moins sociaux (lapin et rat-taupe par exemple) montre effectivement que les espèces eusociales vivent beaucoup plus longtemps. Le rat-taupe nu peut par exemple vivre jusque 30 ans, presque 10 fois plus que la plupart des rongeurs de sa taille. Chez les insectes eusociaux, les reines peuvent même atteindre une longévité 100 fois supérieure. Mais il faut aussi noter qu'il existe des différences de longévité au sein de ces espèces eusociales entre les différentes castes. Par exemple chez la fourmi noire des jardins, la longévité des reines atteint un record de 28 ans, là où les records pour les ouvrières sont plutôt aux alentours de 3-4 ans et les mâles, quant à eux, ne vivent que le temps de la reproduction. L'avantage de l'eusocialité en terme de longévité semble donc davantage porté par les reines que les ouvrières chez les insectes eusociaux. Ceci nous amène à nous poser la question des variations individuelles de longévité selon la place occupée par un individu dans l'organisation sociale de son espèce.

### 2.2. Les conséquences de la socialité sur la longévité à l'échelle des individus

Chez les espèces sociales, la simple absence d'interaction avec des congénères (= isolation sociale) peut avoir des conséquences dramatiques : déclin cognitif chez la souris, raccourcissement des télomères chez le perroquet, augmentation de la concentration d'hormone du stress chez certains poissons, oiseaux et mammifères dont les humains, et cause même la mort d'ouvrières chez les fourmis. Pour les espèces ayant une organisation sociale avec des rapports hiérarchiques, lorsque la hiérarchie est stable les individus subordonnés sont les plus stressés, mais lorsqu'elle est instable, ce sont chez les individus dominant que l'on mesure le plus de stress oxydant. Chez les espèces eusociales, chaque individu a une place déterminée dans l'organisation sociale de la colonie : la caste. Celle-ci détermine le répertoire comportemental mais aussi la longévité d'un individu. En effet, les individus reproducteurs peuvent vivre jusque 10 fois plus longtemps que les ouvrières. Même dans l'environnement protégé du laboratoire, les grandes ouvrières (dites majors) de la fourmi *Oecophylla smaragdina* meurent plus

rapidement que les plus petites (dites minors), indiquant que la vitesse de vieillissement est dépendant de la caste, même chez les ouvrières. Une influence encore plus forte de la caste a été démontré chez l'abeille domestique (*Apis mellifera*). Chez l'abeille, les jeunes ouvrières réalisent d'abord des tâches à l'intérieur du nid, puis plus vielles, elles deviennent fourrageuses et vont chercher la nourriture à l'extérieur du nid. Il est possible de forcer les vieilles fourrageuses à redevenir domestiques et cette opération redonne aux vieilles ouvrières des caractéristiques de jeunes ouvrières, notamment en restaurant leurs capacités cognitives et rétablissant le bon fonctionnement de leur système immunitaire. Le rôle social peut donc chez cette espèce aller jusqu'à réparer les dégâts causés par le vieillissement.

### B) Objectifs et organisation de la thèse

Nous avons vu d'une part qu'il est indéniable que le rôle d'un individu (p. ex. dominant chez une espèce hiérarchique, ouvrière chez un insecte social) impacte ses propres mécanismes de vieillissement ou ceux des autres (p. ex. conflits pour la hiérarchie). Cependant, nous manquons encore d'études interspécifiques dans des groupes variés pour tirer des conclusions définitives sur les avantages procurés par un plus haut degré de socialité. Dans l'état actuel de nos connaissances, seule l'eusocialité semble prodiguer des bénéfices non controversés en termes de longévité. De plus, les mécanismes cellulaires et moléculaires permettant ces avantages ne sont pas encore clairement élucidés. C'est à ces deux questions que s'intéresse ma thèse dont l'objectif principal est d'explorer l'influence de l'organisation sociale sur les mécanismes du vieillissement. L'objectif est double. En premier lieu, il s'agit de mieux comprendre les liens, peu explorés jusqu'à présent, entre longévité et organisation sociale. Par ailleurs, nous avons vu que les mécanismes proximaux à l'origine de la sénescence des organismes sont encore discutés. Le second objectif est donc de tester et d'explorer les mécanismes cellulaires et moléculaires dans différents taxa afin de compléter notre compréhension, encore très partielle, des phénomènes complexes liés au vieillissement des organismes.

Selon la nature et la complexité de l'organisations sociale d'une espèce, l'influence de celle-ci peut prendre des formes différentes. Ainsi, le **Chapitre 1** s'intéresse au diamant mandarin (*Taeniopygia guttata*), une espèce d'oiseau subsociale. Chez cette espèce, avec un faible degré de socialité, le contexte social varie essentiellement en fonction du nombre d'individus qui peuvent interagir entre eux. Nous avons donc modifié la densité sociale en situation contrôlée et observé les effets sur la physiologie des individus, notamment par la mesure de la longueur des télomères et de la balance oxydative. Puis, le **Chapitre 2** s'intéresse à une organisation sociale plus complexe : l'élevage coopératif des jeunes chez le républicain social (*Philetairus socius*). Cette espèce construit de très grands nids communaux comportant des chambres au sein desquelles s'installent un couple. De tels nids peuvent accueillir plus d'une

centaine de couples. Certains jeunes adultes, appelés auxiliaires, ne se reproduisent pas et assistent les parents dans l'élevage des poussins. L'influence des auxiliaires sur le succès reproducteur et la survie des parents a déjà fait l'objet de nombreuses études qui tendent à démontrer un avantage de ce mode de reproduction pour les parents. En revanche, l'influence des auxiliaires sur les jeunes qu'ils aident à élever est encore peu étudiée, notamment en ce qui concerne la survie et la longévité. L'étude présentée dans ce chapitre analyse l'effet de la présence des auxiliaires avant et / ou après l'éclosion sur la longueur des télomères et la survie à moyen terme des poussins. Ces deux premiers chapitres, nous permettent donc d'appréhender l'influence de la socialité sur le vieillissement chez des modèles animaux classiques (oiseaux) et via des marqueurs du vieillissement bien établis dans ce groupe taxonomique (balance oxydative et longueur des télomères). Après l'étude d'une espèce subsociale, puis d'une espèce avec élevage coopératif, l'étape suivante dans la complexité de l'organisation sociale est l'étude d'espèces eusociales. Plusieurs études ont montré que les mécanismes habituellement associés au vieillissement ne semblaient pas toujours fonctionner de la même manière chez les insectes eusociaux (termites, abeilles, fourmis, guêpes), ce qui en font de bons candidats pour trouver de nouveaux mécanismes du vieillissement. Ainsi, du fait de leur haut niveau de socialité et des exceptions qu'ils semblent constituer vis-à-vis de certains mécanismes du vieillissement, les insectes eusociaux répondent parfaitement aux objectifs de cette thèse. Les études s'intéressant au vieillissement chez les insectes eusociaux sont généralement focalisées sur très peu d'espèce : abeille domestique, fourmi noire des jardins et bourdon pour la très vaste majorité. Le **Chapitre 3** propose d'étudier les différences en termes de marqueurs du vieillissement entre reines et ouvrières chez plusieurs espèces de fourmis dont l'organisation sociale diffère, parfois à la limite de ne plus être eusociale. Les marqueurs de vieillissement choisis sont ceux bien établis chez les Vertébrés : stress oxydant et longueur des télomères. Les trois derniers chapitres (**Chapitres 4, 5 et 6**) ont pour but d'explorer et de mettre en évidence des processus de vieillissement différents de ceux classiquement étudiés et qui pourraient être à l'œuvre chez les insectes sociaux. Pour cela, j'ai étudié les différences l'ensemble des protéines (= protéome) et l'ensemble des métabolites (= métabolome) entre différentes castes de la fourmi noire des jardins (*Lasius niger*). Ces études comptent parmi les premières à utiliser la protéomique et la métabolomique, plutôt que la génomique (étude de l'ensemble du génome) pour explorer les influences de la socialité sur le vieillissement. De plus, contrairement à la majorité des autres études, celles-ci ne se limitent pas à comparer reines et ouvrières mais aussi les ouvrières entre elles.

En résumé, alors que les deux premiers chapitres proposent d'étudier des mécanismes classiques (télomères et stress oxydant) chez des modèles animaux habituels (oiseaux), le troisième chapitre teste ces mêmes mécanismes mais chez les insectes sociaux qui semblent y faire exception. Enfin, les trois

derniers chapitres cherchent chez ces animaux moins habituels, de nouveaux mécanismes pouvant participer au vieillissement des organismes. Chacun des chapitres présentés ci-dessus est en lien avec un article publié, en cours de publication ou en cours de rédaction.

**C) Résumé des résultats principaux**

Grâce à des études complémentaires et diversifiées (observations comportementales, dosage du stress oxydant, mesure de la longueur des télomères par qPCR, protéomique, métabolomique), la présente thèse a montré que la socialité joue un rôle sur le vieillissement à de nombreux niveaux. Chez le diamant mandarin, le stress social provoqué par l'agression des congénères induit un stress oxydatif et réduit la longueur des télomères chez l'adulte. Et les individus de faible rang hiérarchique semblent les plus touchés par ces conséquences délétères pour leur longévité. Chez le républicain social, la présence d'individus adultes auxiliaires qui aident les parents à élever les jeunes (élevage coopératif) ont une influence positive sur la longueur des télomères des jeunes après leur naissance. En revanche, avant la naissance nous n'avons pas observé d'effet positif, voire au contraire plutôt négatif. Ceci pourrait être dû au fait que les mères investissent moins dans leurs œufs en présence de helpers. Ce qui leur permet de s'économiser et d'avoir par exemple une ponte supplémentaire. De plus, grâce à un suivi annuel des colonies, nous avons pu montrer que la taille des télomères mesurée 9 jours seulement après l'éclosion permettait de prédire la probabilité de survie des jeunes 5 ans après leur envol. Ceci montre donc que l'environnement social (ici présence d'auxiliaire postnataux) est d'une importance cruciale pour la survie à moyen terme des poussins. Enfin, grâce à l'étude des reines et ouvrières de 10 espèces de fourmis, nous avons pu montrer que plus une espèce avait un fort niveau de socialité, plus les reines de cette espèce vivaient longtemps. Le fait que nous ayons trouvé des relations positives entre socialité et longévité uniquement chez les reines et pas chez les ouvrières est en accord avec une étude récente de Lucas et Keller (2020) qui a conclu que les bénéfices de la socialité sont plus sensibles pour les niveaux élevés de socialité et particulièrement chez les individus reproducteurs (ici les reines).

En plus de faire le lien entre certaines organisations sociales et le vieillissement, les études présentées dans ce manuscrit, ont aussi contribué à la meilleure compréhension des mécanismes moléculaires du vieillissement (deuxième objectif de la thèse). Par exemple, chez le diamant mandarin, nous avons pu établir une chaîne causale entre le stress social, le stress oxydant et le raccourcissement des télomères. Le rôle des télomères comme prédicteur de la survie de la progéniture a été confirmé (sur au moins 5 ans) chez le républicain social. Cependant, ce lien n'était pas vrai chez les reines fourmis où celles à la plus longue durée de vie étaient celles avec les télomères les plus courtes. Ce qui va certes à l'encontre du lien positif entre longueur des télomères et longévité observé chez les mammifères et

oiseux, mais rejoint d'autres études réalisées chez les insectes sociaux. Nous avons aussi montré que parmi les reines d'espèces strictement eusociales les reines qui vivaient le plus longtemps avaient en moyenne moins de stress oxydant. Les études protéomiques et métabolomiques ont montré la présence de nombreuses molécules anticancéreuses chez les ouvrières de la fourmi noire des jardins. Or, il est déjà connu que les mammifères qui vivent le plus longtemps expriment aussi une grande quantité et une grande diversité de molécules anticancéreuses. Ceci pourrait donc montrer des convergences évolutives d'espèces très éloignées mais devant faire face à des problématiques similaires (augmentation de la probabilité de développer un cancer en vivant plus longtemps). Les études de protéomique et de métabolomique ont aussi mis en évidence que, comparées aux ouvrières, les reines exprimaient moins de protéines liées à certaines fonctions, par exemple le système immunitaire. Sachant que la structure sociale de la colonie protège, au moins en partie, la reine des pathogènes (mécanisme connu sous le nom d'immunité sociale), nous avons fait l'hypothèse qu'elle peut alors se permettre d'investir moins d'énergie dans son système immunitaire au profit de la reproduction et de la longévité. Toujours grâce aux études de protéomique et métabolomique, nous avons pu déceler chez la fourmi noire des jardins des molécules appartenant aux voies de signalisation comme celles des sirtuines ou de mTOR. Or, ces voies sont connues pour moduler la longévité selon l'état énergétique de l'organisme. Selon les études réalisées à ce jour, ces voies de signalisation sont parmi les premières à pouvoir ralentir les effets du vieillissement et à prolonger l'espérance de vie. Cependant, des études spécifiques doivent être menées pour comprendre leur régulation fine et ainsi évaluer l'universalité de ces mécanismes dans le vieillissement animal. Pour finir, chez la fourmi noire des jardins, ainsi que chez beaucoup d'autres espèces de fourmis, le passage d'une caste spécialisée à l'autre est fortement dépendant de l'âge. Dans le Chapitre 6, nous avons donc mis en place un protocole nous permettant d'avoir quatre groupes d'ouvrières différents par l'âge (1 mois ou 1 an) et la caste (domestique ou fourrageuse). Grâce à cette expérience, nous avons pu montrer que la physiologie des ouvrières était à la fois influencée par l'âge et la caste. L'effet âge se traduisait par le fait que les jeunes utilisaient préférentiellement des lipides alors que les vieilles plutôt les glucides. Il y avait aussi moins de molécules permettant la détoxification de l'organisme chez les individus plus âgés, quelle que soit la caste. L'effet caste principal, aussi retrouvé dans les deux autres études de protéomiques et métabolomique, était que les domestiques (ouvrières qui restent à l'intérieur de la colonie) exprimaient beaucoup plus de molécules liées à la digestion et à l'assimilation des nutriments.

**D) Axes d'amélioration pour de futures études liant vieillissement et longévité**

L'analyse critique de la thèse, réalisée notamment grâce aux membres du jury a permis de dégager des points des axes de recherche future pour mieux comprendre les liens entre vieillissement et longévité.

**1. Comparer la variation de socialité chez d'autres insectes.** Dans ma thèse, j'ai mené une étude interspécifique chez 10 espèces de fourmis. Même si le degré d'eusocialité peut varier entre les espèces elles restent eusociales. En revanche, il existe des espèces de guêpes qui sont strictement solitaires et d'autres strictement eusociales. Comparer ces espèces aux antipodes de la socialité permettrait de renforcer les contrastes biologiques et de mieux mettre en évidences les mécanismes impliqués dans une plus grande longévité des espèces eusociales.

**2. Trouver des points de comparaison entre Vertébrés et Invertébrés.** Au cours de ma thèse, j'ai étudié des Vertébrés (oiseaux) et des Invertébrés (fourmis). Cependant, je n'avais pas de point de comparaison entre ces espèces qui m'auraient permis d'évaluer si pour une organisation sociale donnée, les mécanismes de vieillissement associés divergent entre Vertébrés et Invertébrés. Cela aurait été possible en étudiant la hiérarchie chez des espèces de fourmis primitives comme *Dinoponera quadriceps* où l'accès à la reproduction des ouvrières se fait selon le rang hiérarchique de celles-ci, rang qui s'obtient en remportant des combats. Ce système est très ressemblant à ce qui peut se retrouver chez des espèces de mammifères, à la différence près que chez les mammifères se sont généralement les mâles qui sont en compétition pour l'accès à la reproduction.

**3. Mettre au point des expériences précises suite à la protéomique et à la métabolomique.** De par leur nature exploratoire, ces méthodes restent spéculatives. Nous mettons en évidences des différences de quantité de protéines ou de métabolites, d'après la littérature ces molécules sont impliquées dans telle ou telle fonction, alors nous en déduisons que cette fonction est impliquée dans les différences observées entre échantillons. Ainsi, les méthodes exploratoires permettent d'ouvrir de nouvelles pistes qu'il faut ensuite soumettre à une expérience rigoureuse, quantifiées. Par exemple, si les reines ont moins de molécules liées au système immunitaire, il faudrait soumettre les reines et les ouvrières à un pathogène et mesurer leur résistance à ce pathogène. De même, la présence de nombreuses molécules anticancéreuses est prometteuse mais il faut mesurer la capacité des fourmis à ne pas développer de tumeurs avant d'affirmer que les fourmis, comme le rat-taupe nu (mammifère eusocial) sont immunisées contre le développement de cancers.

# List of publications and oral presentations

## Articles published in peer-reviewed journals

1. **Martin Quque**, Margaux Benhaim-Delarbre, Fabrice Bertile, Jean-Louis Deneubourg, Cédric Sueur, François Criscuolo (2019). *Division of labour in the black garden ant (Lasius niger) leads to three distinct proteomes*. Journal of Insect Physiology, **117**(103907). Doi:10.1016/j.jinsphys.2019.103907
2. **Martin Quque**, Olivier Bles, Anaëlle Bénard, Amélie Héraud, Bastien Meunier, François Criscuolo, Jean-Louis Deneubourg, Cédric Sueur (2020). *Hierarchical networks of food exchange in the black garden ant Lasius niger.* Insect Science. Doi:10.1111/1744-7917.12792

## Articles submitted in peer-reviewed journals

3. **Martin Quque**, Rita Covas, Matthieu Paquet, Sandrine Zahn, Cédric Sueur, François Criscuolo, Claire Doutrelant. *Post-hatching helper effects increase early telomere length in a cooperatively breeding bird*. (in *review in Oecologia*)
4. Cédric Sueur, **Martin Quque**, Alexandre Naud, Audrey Bergouignan, François Criscuolo. *Network age: a new dimension in healthy ageing.* (in review in Trends in Ecology and Evolution)

## Articles in draft form

5. **Martin Quque**, Charly Ferreira, Sebastian Sosa, Quentin Schull, Sandrine Zahn, François Criscuolo, Josefa Bleu, Vincent A Viblanc. *Domino effect of conspecific aggression on oxidative status and telomere length in zebra finch*.
6. **Martin Quque**, Claire Villette, François Criscuolo, Cédric Sueur, Fabrice Bertile, Dimitri Heintz. *A metabolomics study of task specialization in a social insect, the black garden ant (Lasius niger)*.
7. **Martin Quque**, Charlotte Brun, Claire Villette, François Criscuolo, Cédric Sueur, Fabrice Bertile, Dimitri Heintz. *Combining metabolomics and proteomics to disentangle age and caste effects in black garden ant workers (Lasius niger)*
8. **Martin Quque**, Cédric Sueur, Sandrine Zahn and François Criscuolo. *Soma maintenance theories of ageing in the light of ants' lifespan diversity*.

## Chapters published in books

9. **Martin Quque**, Olivier Bles. (2020) Lasius. In: Starr C. (eds) Encyclopedia of Social Insects. Springer, Cham. Doi:10.1007/978-3-319-90306-4

## Conferences attended (English-speaking)

1. **Martin Quque**, Fabrice Bertile, Dimitri Heintz, Cédric Sueur, François Criscuolo (2019). *Exploring the links between ageing and sociality in ants by the means of omics methods and oxidative status assessment.* Seminar of the Jürgen Heinze's team, Regensburg, Germany. Oral communication

2. **Martin Quque**, Margaux Benhaim-Delarbre, Fabrice Bertile, Jean-Louis Deneubourg, Cédric Sueur, François Criscuolo (2019). *Task specialization in the black garden ant (Lasius niger) leads to three distinct proteomes.* Central Europe IUSSI meeting 2019, Vienna, Austria. Oral communication

3. **Martin Quque**, Margaux Benhaim-Delarbre, Fabrice Bertile, Jean-Louis Deneubourg, Cédric Sueur, François Criscuolo (2019). *A proteomic insight into life-history trade-offs in social insects.* Young Natural History scientist Meeting (YNHM) 2019, Paris, France. Oral communication

4. **Martin Quque**, Margaux Benhaim-Delarbre, Fabrice Bertile, Jean-Louis Deneubourg, Cédric Sueur, François Criscuolo. *How can social organisation explain the differences in longevity in the black garden ant (Lasius niger)?* Colloque d'ÉcoPhysiologie Animale 2017, Strasbourg, France. Oral communication

# Abbreviation and acronyms

| | |
|---|---|
| **Cq, Ct, Cp** | cycle number threshold: number of amplification cycle of qPCR needed to have a detectable signal |
| **CTAB** | hexadecyltrimethylammoniumbromid |
| **DNA** | deoxyribonucleic acid |
| **insulin / IGF pathway** | insulin/insulin-like growth factor pathway |
| **LWRh** | long-wavelength rhodopsin |
| **mTOR** | mechanistic target of rapamycin |
| **PCI** | solution of phenol, chloroform and isoamyl alcohol (25:24:1) |
| **PCR / (q)PCR** | (quantitative) polymerase chain reaction |
| **PVP** | polyvinyl pyrrolidone |
| **ROS** | reactive oxygen species |
| **RT** | room temperature |
| **b-ME** | beta mercaptoethanol |
| **FDR** | false discovery rate |
| **MLSP** | maximum life span potential |
| **RNA** | ribonucleic acid |
| **rTL** | relative telomere length: used to qualify the measure of telomere length by quantitative polymerase chain reaction |
| **SOD** | superoxide dismutase (an antioxidant enzyme) |
| **TL** | telomere length |

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Chaque fleur qui tombe
Les fait vieillir davantage
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Yosa Buson

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Échappe à ses claques
Ah ! cette main ridée.

The flock of flies
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Ah! That wrinkled hands.

Kobayashi Issa

Rien ne dit
Dans le chant de la cigale
Qu'elle est près de sa fin.

No warning
In the cicada's song
But the end is close.

Matsuo Basho

## Why and how do we grow old to the end?

### *Historical roots of ageing understanding*

Why and what do we die of when death occurs naturally? This is, perhaps, one of the oldest questions humanity has ever asked itself. In antiquity[1], death, old age and related diseases were inevitable, stemming from the will of gods, often inflicted on men to punish a fault (ancient myths of Prometheus, Gilgamesh, Adam and Eve). In the tradition of Empedocles, Aristotle imagined that the physical world was governed by the interactions of four primordial elements: water, earth, air, and fire. In this context, he saw the passage from youth to old age as a gradual change of the organism from a warm and moist state (childhood) to a warm and dry state (adulthood) and finally to a cold and dry state (old age). In the 2nd century A.D., Galen hypothesised that this age-inducing dryness began at conception because of the sperm heat. He also hypothesised that this drying up could be beneficial in early adulthood but would become harmful later in life. Although the idea of an "ageing dryness" has been left aside, the idea that the same process could have opposite consequences according to the period of life was taken up again much later in the 20th century by George C. Williams (1957) under the name of *antagonistic pleiotropy*. Today, this principle is still part of the theories trying to explain the origins of ageing. While some Arab and European physicians and philosophers of antiquity such as Aristotle, Epicurus and Avicenna saw old age and death as not only inevitable but even necessary to maintain the Universe's equilibrium, not everyone shares this fatalistic view. Others believe in the possible extension of human life, also called prolongevity of life. Notably, in the eighth century A.D., the alchemist Al Jabir wrote many texts that spread in the Arab world and in Europe Taoist precepts to achieve extreme longevity and even immortality. Later, the English philosopher and scientist Roger Bacon (1214-1294) and the Italian writer Luigi Cornaro (~1460-1566) will contribute strongly to spread in Europe the idea of the possibility of extending the human life span, beyond 100 years and even 900 years for Bacon. To achieve this longevity, both advocated a healthy lifestyle without excess and stressed the importance of diet. During the eighteenth century, progress in medicine and science in general led the prolongevists to gradually abandon the beliefs inherited from antiquity and turn to a more scientific study of the causes of disease and old age.

[1] This first historical paragraph uses two main references: the first chapter of Carnes and Olshansky's book "The Quest for Immortality: Science at the Frontiers of Ageing" (2002) and Gruman's very detailed book "A history of ideas about the prolongation of life: the evolution of prolongevity hypotheses to 1800" (1966). The reader who wants to deepen the historical part which is not the main object of my thesis can find these references in the bibliography at the end of the manuscript.

Since then, humanity has accumulated a great deal of knowledge and has made great technological progress. Nevertheless, ageing, which is defined in biology as a progressive loss of an organism's ability to maintain its vital functions over time, has not yet revealed all its secrets. In light of the theory of evolution of species through natural selection, the very existence of biological ageing seems paradoxical. According to this theory, living beings are selected on their ability to maximize the transmission of their genetic characteristics to subsequent generations. They must therefore survive long enough to be able to reproduce and breed with the best possible success rate. Individuals best optimizing these two parameters (survival and reproduction) are said to have greater fitness. However, biological ageing, also known as senescence, has a negative effect on these two parameters. Indeed, living beings experience a loss of efficiency of the functions essential to their survival with age. Age-related loss of muscle strength and endurance (i.e. sarcopenia, see Greenlund and Nair 2003 for further details in humans) is widespread in animals: horses (Mota *et al.* 2005), dogs (Täubert *et al.* 2007), rodents (Punzo and Chavez 2003), birds (Costantini *et al.* 2008b), fishes (Reznick *et al.* 2004), and insects (Schumacher *et al.* 1997). This deterioration in physical ability makes the prey less able to escape the predator (Slobodkin 1968; Veasey *et al.* 2000) and the predator less able to catch its prey (Holekamp *et al.* 1997; Täubert *et al.* 2007; MacNulty *et al.* 2009). Immune defences also decrease with age, increasing the risk of dying from a pathogen in older individuals. This phenomenon known as immunosenescence is well-established in vertebrates (see Peters *et al.* 2019 for a meta-analysis in birds, mammals and reptiles) but requires further studies in invertebrates, where results are currently equivocal (Moret and Schmid-Hempel 2000; Bocher *et al.* 2007; Schmid *et al.* 2008; Armitage and Boomsma 2010, reviewed in Stanley 2012) and potentially related to the reproductive status of individuals (Adamo *et al.* 2001; Rolff 2001; Rolff and Siva-Jothy 2002). As with the ability to survive, reproductive success decreases with age (Mangel and Heimpel 1998; Tatar 2010; Lemaître and Gaillard 2017). Here, the exception of menopause in some mammalian species should be mentioned. Humans (*Homo sapiens*), pilot whale (*Globicephala macrorhynchus*) and killer whale (*Orcinus* orca) have a particularly long post-reproductive life (after menopause). Several studies in humans (e.g. Hawkes 2003; Lahdenperä *et al.* 2004; Shanley *et al.* 2007) and another very detailed one in killer whales (Brent *et al.* 2015) have shown that post-menopausal females, thanks to their comprehensive knowledge of the environment acquired during their lifetime, ensure greater survival of their grandchildren. In this very particular case, ageing is associated with an indirect improvement in reproductive success. This phenomenon is referred to as the grandmother hypothesis. In elephants, the grandmother presence in the same herd, regardless of her reproductive status, decreases the inter-birth interval of its daughters and enhances calf survival probability (Lahdenperä *et al.* 2016). This observation suggests that grandmothers might benefit their daughters and grandchildren even in species that do not exhibit a long post-reproductive lifespan. In order to underline the

universality of senescence in pluricellular organisms, it can be noted that plants also see their biological functions become less efficient with age: lower efficiency in chlorophyll synthesis (Akoyunoglou and Argyroudi-Akoyunoglou 1969), in net energy assimilation (Thorne 1960), or poorer seed quality beyond a certain age (Lyngdoh *et al.* 2014). With the knowledge of the adverse effects mentioned above, it is therefore understandable that the slightest mutation that would allow the phenomenon of ageing to be overcome would be a powerful evolutionary advantage and would make individuals carrying this mutation very competitive in the struggle to survive and spread their genes. How can it be explained that natural selection has not rid living beings of such a handicap to their fitness? Two possibilities arise: either it is a universal constraint that cannot be avoided, or its impact on fitness escapes natural selection. Some examples of animals show us that it is in fact possible to escape from ageing. Among these species, the most impressive is certainly the jellyfish *Turritopsis nutricula* which, even after reaching sexual maturity, is able to return to an earlier form of its development, the polyp stage, and then resume normal development (Piraino *et al.* 1996), making it potentially immortal, extrinsic mortality factors (*e.g.* pathogens, predators) being left aside. Such rejuvenation has been observed in the laboratory up to ten times for the same individual (Kubota 2011). However, compared to the 1.2 million of known eukaryotic species (Mora *et al.* 2011), only very few show such an ability. It remains to be explained how in 3.5 Ga of the evolutionary history of Life on Earth (Van Kranendonk *et al.* 2008), ageing and subsequent death continue escaping natural selection without being more effectively counter-selected in all living beings.

### *Evolutionary theories of ageing*

Like any gene, those causing senescence can be eliminated only if selection takes place before transmission to offspring, *i.e.* before or during the reproductive life of the individuals[2]. Therefore, the hypotheses below have in common to explain how the genes involved in senescence can elude this time window of selection. One of the first modern theories of the maintenance of senescence in the evolutionary history of living organisms is the so-called *accumulation of mutations,* developed by Medawar and Haldane (Haldane 1933; Medawar 1952). This theory postulates that animals have a high chance of dying due to extrinsic factors, such as predation or pathogens, long before dying from senescence. The selective pressure would therefore be much more in favour of mechanisms reducing predation or infection risks than in favour of mechanisms to maintain the organism over the long term. DNA (deoxyribonucleic acid) mutations and damage to other macromolecules therefore accumulate

[2] As far as we know, only very rarely after, as illustrated previously in the very particular case of cultural transmission of ecological knowledge by old female killer whales, humans, or elephants to their children and grandchildren.

over the lifetime of the individual without being sufficiently repaired and eventually cause senescence of the organism. In this theory, senescence appears as a secondary product of the organism's pace of life.

This theory can be contrasted with the fact that senescence is a continuous process that begins early in an individual's life. Thus, even a slight weakening of the ability to survive and/or reproduce can take place while the individual is still able to reproduce. To explain why such senescence genes are still selected, George C. Williams (Williams 1957) proposes another theory called *antagonistic pleiotropy*. Pleiotropy is the ability of a gene to code for several different phenotypes, especially during the individual's lifetime. The gene would first offer a reproductive advantage, resulting in its positive selection, then negative effects would emerge after the reproductive life of the individual and the gene could no longer be eliminated, despite the intervention of natural selection. This is, for example, what is observed in the genes coding for the Nox and Duox families of enzymes. These enzymes are involved in signalling pathways related to immunity, but they are also implicated in the development of chronic diseases at the end of life such as hypertension, atherosclerosis, and Alzheimer (Lambeth 2007).

Kirkwood added to these two previous theories the notion of energy trade-offs, particularly between the soma and the germline, in a non-exclusively genetic theory that later became known as *disposable soma* (Kirkwood *et al.* 1979). This hypothesis is based on the observation that the resources available in the environment are limited. The reproductive function is therefore in competition for energy resources with the survival and growth of the organism. Selective pressure would therefore initially be exerted on the mechanisms for escaping extrinsic mortality factors: *e.g.* immune system, development of the ability to feed or escape from predators. Then, once the reproductive system is functional, energy resources would be allocated primarily to the germline in order to optimize reproductive success, but at the expense of protection and repair of the soma. This defect in energy allocation triggers the accumulation of damage and progressive decrease of vital functions: senescence.

The three theories presented above interpret ageing as the unfortunate consequence of other processes (accumulation of damage over time, pleiotropy, energy trade-offs), but not as a true evolutionary process, selected for its own sake. Nevertheless, some authors ask this question: is it possible to have adaptive ageing, selected for evolutionary benefits? In the tradition of Avicenna, Aristotle and Epicurus, group selection (Smith 1964; Wilson 1997; Shanahan 1998) was first cited to think about death as a useful phenomenon: older individuals die to allow younger ones to benefit from resources, the individual death is then altruistic to the benefit of the group. This notion has always been

part of the collective imagination, as evidenced by the ancient accounts of ritual senicides[3] in Japan (*ubasute*) or Scandinavia (*ättestupa*)[4]. However, no simulation has been able to demonstrate a beneficial influence of group selection on the emergence of ageing as an altruistic process, especially because the cost of death at the individual level is too high compared to the potential benefits, which are diffuse at the group level (Longo *et al.* 2005). For this reason, the hypothesis of programmed altruistic ageing was initially quickly abandoned. More recently, several studies propose to reconsider this hypothesis (Mitteldorf 2004, 2012; Longo *et al.* 2005). They argue that local extinctions of populations due to acute starvation are a selection pressure strong enough to influence the selection of altruistic ageing mechanisms to stabilise population size. Among the other proposed evolutionary benefits associated with ageing and death are the ideas that generational turnover increases genetic diversity and fosters the selection of favourable traits (Skulachev 1997; Goldsmith 2008; Yang 2013). Although some evidence of this beneficial ageing, also known as adaptive or altruistic ageing, appears to have been provided in *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Mus musculus* (Longo *et al.* 2005), particularly when the environment quickly changes (Herrera *et al.* 2017), in the current state of knowledge it seems to result more from particular conditions, rather than being a rule followed by the majority of living beings (Kirkwood and Melov 2011).

These hypotheses, which are not mutually exclusive (**Figure 1**), define the theoretical evolutionary framework of the biology of ageing. This theoretical framework makes it possible to imagine how senescence can escape natural selection despite the strong evolutionary disadvantages it brings. However, that does not explain how and through which biological processes organisms age. Such proximal mechanisms are detailed in the next section and **Figure 2** proposes an illustrative overview of them.

### *Proximal mechanisms*

In a comprehensive review of the literature, López-Otín et al. (López-Otín *et al.* 2013) identified and classified nine hallmarks of ageing caused by mechanisms I will describe in this section, but also involving epigenetic mechanisms that I will not detail here, as I did not have the opportunity to test them during my PhD. These nine hallmarks of ageing are genomic instability, telomere erosion, epigenetic alterations, loss of proteostasis (*i.e.* the proper functioning of protein metabolism from synthesis to degradation), dysregulation of the nutrient detection system, mitochondrial dysfunction, cell senescence, stem cell exhaustion and impaired intracellular communication.

[3] Aka. geronticides, the killing (or suicide) of the elderly.
[4] Current research since the 20th century have brought evidences that these two rituals actually seem to pertain more to myth than reality (Battin 1987; Odén 1996).

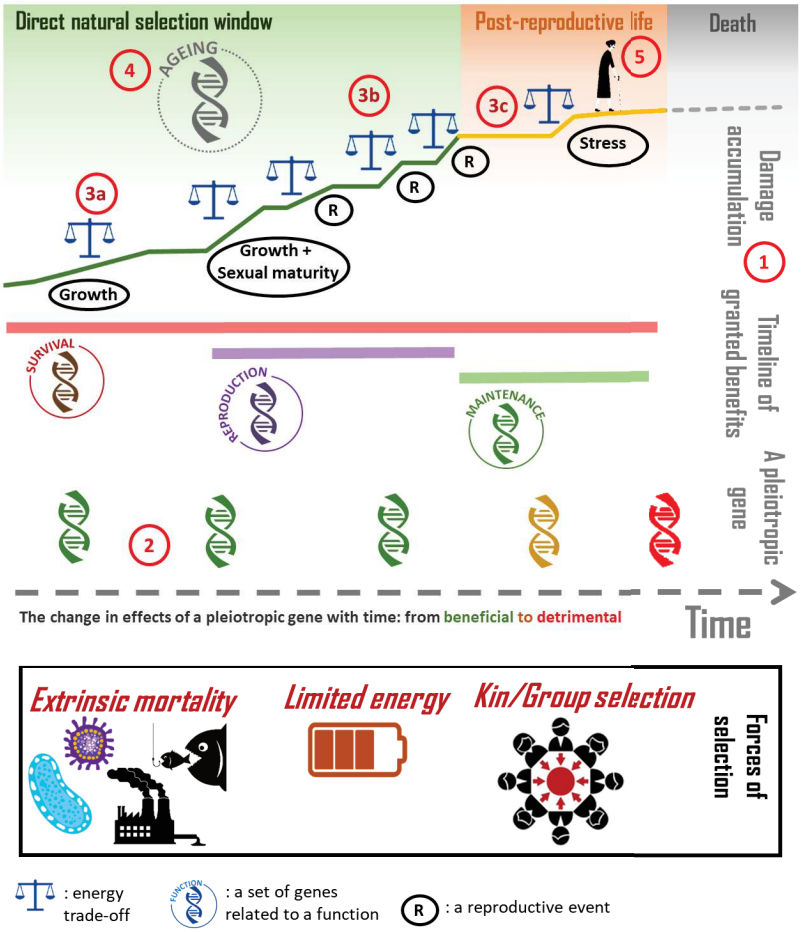


Figure 1: **Evolutionary theories of ageing and natural selection forces on species longevity.** While senescence strongly decreases fitness by decreasing both reproductive success and probability of survival, it has not been effectively counter-selected over the evolutionary history of life on Earth. Therefore, either it is an inevitable cost of life, or senescence can escape natural selection. The example of the jellyfish *Turritopsis nutricola*, which is capable of reversing the ageing process and returning to a state of development prior to sexual maturity, shows us that certain organisms seem to be able to free themselves from ageing. This figure illustrates the evolutionary theories of ageing giving the theoretical framework to explain how ageing and death escape selection. ① **Mutation accumulation.** Extrinsic mortality (e.g. predation, exposure to pathogens or pollutants) favours genes related to survival and reproduction, because wild animals die before the consequences of accumulated damage can be felt, preventing the selection of genes related to somatic maintenance, which in this case provides no benefit. This sheds light on the importance of extrinsic mortality in the selection of species with shorter or longer lifespan. Indeed, a lightened effect of extrinsic mortality (e.g. favourable environment or efficient anti-predation features) allows the selection of mechanisms to repair the damage in the long term. ② **Pleiotropic antagonism.** Some genes, useful in the early life may become detrimental later in life. Because of their early advantage, when the selection pressure is the strongest, they remain selected despite their late deleterious effects. This is for example the case of genes coding for the enzyme family Nox and Duox (Lambeth 2007) ③ **Disposable soma theory.** Because of limited energy resources, an individual who maximised reproduction and survival early in life invested less in molecular protection mechanisms. This hypothesis, not exclusively genetic unlike the previous ones, can be generalised at any time in individual life history: because of limited resources and depending on the context, stress may induce ageing (3c, see main text), reproduction may occur at the expense of immunity (Capilla-Lasheras 2017), growth at the expense of longevity (3a, Monaghan and Ozanne 2018), reproduction at the expense of longevity (3b, Sudyka 2019), immunity at the expense of digestion (Adamo et al. 2010). ④ **Adaptive ageing theory (altruistic ageing).** In contrast to the previous three hypotheses, this theory proposes to see ageing as an evolutionary advantage actively selected during evolution for the benefits it provides at the group level (genetic diversity, stabilization of population sizes to avoid starvation, promotion of adaptive mutations). This theory, which is based on kin selection and group selection, is still poorly studied and supported. Nevertheless, several studies from the 2000s (e.g. Mitteldorf et al. 2012) propose theoretical evolutionary framework. ⑤ **Grandmother theory.** In some species (killer whales, pilot whales and humans), females have a particularly long post-reproductive life (menopause). A long menopause would be explained by the transmission of ecological knowledge from old females to their children and grandchildren (increase in inclusive fitness). This is one of the rare cases where the natural selection window extends beyond the reproductive life of the individual.

The very first hypothesis to propose a precise cellular mechanism was theorised by Denham Harman in the 1950s as the *free radical theory of ageing* (Harman 1955, 1992). Chemically, a (free) radical is a chemical species with unpaired electrons on the outermost shell. This characteristic makes free radicals very likely to interact with other molecules and take electrons from them to complete their outermost shell. The target molecule is then oxidised by the free radical. Mitochondria has special importance among the primary endogenous source of free radicals in animals. It provides the cell with energy via the formation of ATP (adenosine triphosphate), in particular through several molecules forming an electron transport chain (Jin and Bethke 2002) the final element of which is oxygen ($O_2$). Even during the normal operation of this transport chain, an electron may escape and react with oxygen, first forming the superoxide anion $O_2^{\bullet -}$, then hydrogen peroxide $H_2O_2$, which crosses the mitochondria's membranes more easily and has a longer half-life. This will propagate the oxidative wave outside the mitochondria (Miquel *et al.* 1980; Starkov 2008; Murphy 2009; Wolf 2010 pp. 167–9). All molecules, radical or not, resulting from these oxidation-reduction reactions are called reactive oxygen species (ROS). ROS threaten the integrity of genetic information by inducing numerous errors in both the nuclear (Forsberg *et al.* 2012; Faggioli *et al.* 2012; Moskalev *et al.* 2013) and mitochondrial genomes (Park and Larsson 2011), damage the cell membrane by oxidizing the phospholipids (Ward *et al.* 2005) or disrupt cell function by degrading proteins that, under the action of ROS, can fragment into polypeptides or agglomerate non-functionally (Powers *et al.* 2009; Wolf 2010 p. 184; Koga *et al.* 2011; López-Otín *et al.* 2013).

Since mitochondria are the major source of ROS, one of the first ways to prevent oxidative damage is to reduce their production by mitochondria through the use of decoupling proteins (UCPs). These proteins allow the passage of protons from the peripheral space to the mitochondrial matrix without passing through the ATP synthase (**Figure 2**). This reduces the electrochemical gradient around the inner membrane and the production of ROS which is highly dependent on it (Brand *et al.* 2004; Brand and Esteves 2005). Such protective mechanisms have been observed in organisms as diverse as yeasts, birds or mammals in response to stress and/or increased metabolism (Criscuolo *et al.* 2005; Stier *et al.* 2014). There are also mechanisms of repair (e.g. recycling of defective proteins by the proteasome, DNA repair; see López-Otín *et al.* 2013), and protection mechanisms grouped under the term "antioxidant barrier". This antioxidant defence system can be divided into three components. First, antioxidant enzymes (*e.g.* superoxide dismutase, catalase) that transform ROS into less active derivatives that are more easily eliminated by the body (Michiels *et al.* 1994; Fridovich 1995; Johnson and Giulivi 2005). Conversely, some molecules do not transform ROS but neutralize it (Hanukoglu 2006; Droge *et al.* 2006). They are oxidised but not reactive enough to continue a chain reaction. These molecules are called reducing molecules, they can be hydrophilic and present rather in the cytoplasm (*e.g.* glutathione,

ascorbate) or lipophilic and present rather in the membranes (*e.g.* tocopherols, carotenoids). The last category is made of the so-called regenerative enzymes (*e.g.* thioredoxin, glutathione reductase). They allow lipophilic or hydrophilic reducers to regain their state prior oxidation and thus restore their antioxidant buffering capacity (Nordman *et al.* 2003; Lu and Holmgren 2014).

The ideal culprit of ageing seems to have been found: the activity of the metabolism generates ROS which leads to damage to biological molecules. The explanation is simple, compatible with evolutionary theories of ageing and matches the general tendency of animals with a high metabolism to age faster (incompletely theorised as the rate of living theory by Pearl 1928). Nevertheless, several studies show that ROS, far from being only deleterious molecules, are even essential in certain cellular signalling pathways (Mittler *et al.* 2011), especially for innate immunity (Matsuzawa *et al.* 2005; Kohchi *et al.* 2009; Lam *et al.* 2010). This shows that oxidizing and anti-oxidizing molecules are in fact part of a balance, the two components of which are essential to the proper functioning of the organism. Thus, oxidative stress is not defined as the mere presence of oxidizing molecules but rather as an unbalanced oxidative status in their favour. Moreover, although several correlative studies show a negative association between oxidative stress and longevity (Sohal *et al.* 1993; Agarwal and Sohal 1994; Forster *et al.* 1996), this is not the case for other experimental studies carried out in mice (reviewed in Speakman and Selman 2011). Similarly, clinical trials with antioxidant intake have not been able to demonstrate an increase in longevity (Howes 2006; Bjelakovic *et al.* 2007; Chong *et al.* 2007). In honeybees (*Apis mellifera*), as in black garden ants (*Lasius niger*), the gene coding for the superoxide dismutase (SOD), an antioxidant enzyme, was found to be less strongly expressed in queens, even though they are long-lived (Parker *et al.* 2004; Corona *et al.* 2005; Corona and Robinson 2006). In general, studies in social insects do not agree with predictions derived from the radical theory of ageing (Lucas and Keller 2014). However, these studies have mainly been conducted in honeybees. Further studies are needed to ensure that this trend is found in all social insect species. In conclusion, oxidative stress, although related to the mechanisms of senescence, does not seem to explain all its complexity. So, we need to look for complementary biological mechanisms underlying senescence.

More recently, ageing biologists have become interested in telomeres. Telomeres are repeated sequences at the ends of linear chromosomes. They were discovered in the 1930s par Hermann Muller (1938) and Barbara McClintock (1941). During replication, the lagging strand end of the DNA cannot be replicated because it lacks a primer with a 3'OH end necessary for DNA polymerase III to function (Venkatesan *et al.* 2017). As a result, at each cell division, the ends of the chromosomes shorten.

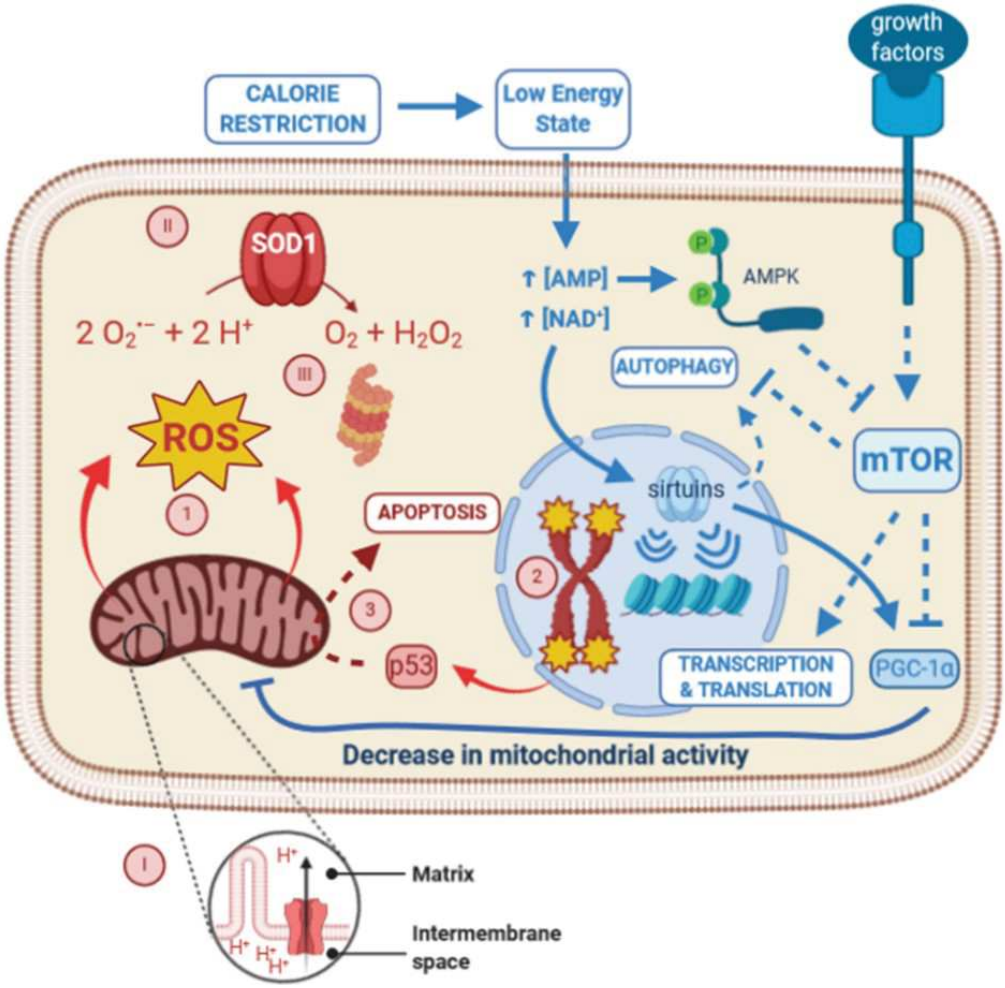


**Figure 2. Proximal mechanisms of ageing and mitigation responses.** On this schematic view of an animal cell, dashed lines indicate that several intermediates are omitted for clarity and legibility purposes, solid lines suggest a more direct link. Arrows with triangular head show an activation, while flat-headed arrows show an inhibition. Representation of molecules are purely illustrative and does not reflect their actual molecular structure. In red tones and rather to the left of the figure, are mechanisms related to the generation of reactive oxygen species (ROS, **1**) in the mitochondria during cell respiration. These highly reactive molecules damage impair macromolecules, such as lipids, proteins, DNA. Among the damaged DNA sequences, telomeres **(2)**, which cap the end of linear chromosomes, are of the greatest interest. Linked to genetic, epigenetic end ecological environment, they have been proved to reflect individual quality and/or life expectancy in several species. The faster they erode, the shorter the lifespan. Their shortening triggers apoptosis **(3)** through the transcription cofactor p53. Animal cells have evolved means to protect themselves from the harmful effect of oxidative stress by: **(I)** preventing the generation of ROS (e.g. mitochondrial uncoupling proteins); **(II)** deactivating ROS (e.g. the cytoplasmic Cu/Zn SOD1 transforms the anion superoxide into $O_2$ and $H_2O_2$); **(III)** repairing or recycling damaged molecules (e.g. the proteasome recycles misfolded proteins). In blue tones and rather to the right of the figure, are mechanisms related to cellular energetic state and metabolic activity. The mToR pathway (mechanistic target of rapamycin) is a metabolism activator, notably by promoting transcription and translation and by inhibition of PGC-1α which reduces mitochondrial activity. In addition, the mToR pathway inhibits autophagy, which has been shown to prevent cell senescence. Growth factors (such as insulin-like growth factor) activate mToR. Conversely, caloric restriction induces a low energy level in the cell which results in increased concentrations of adenosine monophosphate (AMP) and oxidised nicotine amide dinucleotide ($NAD^+$). High concentration of AMP results in the activation of the AMP-dependent kinase which inhibits mToR and its downstream consequences. Increased concentration of $NAD^+$ allows the activation of the NAD-dependent deacetylases, sirtuins. They deacetylate histones and have opposite effects to mToR pathway: autophagy activation and mitochondrial activity inhibition through PGC-1α activation. Hence, the energy state can either favour the mToR pathway, which is conducive to growth and ageing, or the sirtuin pathway, inducing low metabolic activity and a protective state. *Created with BioRender.com*

Telomeres are therefore shortened in place of the coding sequences of the genome. This protective role of telomeres and their structure have been extensively studied by Elizabeth Blackburn, Carol Greider and Jack Szostak (e.g. Yao *et al.* 1981; Szostak and Blackburn 1982; Shampay *et al.* 1984; Greider and Blackburn 1985), who received a Nobel Prize for their work in 2009. After a certain number of divisions, telomeres become too short and can no longer fulfil their protective role. When this stage is reached, the cell then triggers signalling pathways that will lead to its programmed death: apoptosis. This threshold is called Hayflick's limit (see Shay and Wright 2000 for a history of discovery and link with telomeres). It depends on the cell type and the organism under consideration, but it is for example estimated in humans at an average value of about 50 divisions before the onset of apoptosis (Hayflick and Moorhead 1961; Hayflick 1965). Apoptosis triggered by the shortening of telomeres is done via the activation of the p53 transcription factor (Artandi and Attardi 2005). The shorter the telomeres or the faster their attrition, the sooner a cell will die, leading to a decrease in functional cells in the body and thus possible dysfunction. The telomere length appears to fulfil the wish of evolutionary biologists in the early 2000s, who were interested in finding a marker, simple to measure, that would match with the age of individuals in their quest of characterizing the determinants of individual fitness (Hall *et al.* 2004; Monaghan and Haussmann 2006; Pauliny *et al.* 2006). The telomere length at conception appears to be controlled in part by genetic and heritable factors. For example, a study conducted with twins of the same sex (Hjelmborg *et al.* 2015) showed that not only was the telomere length in human leukocytes heritable (64% with 22% shared influence with the environment), but also their shortening rate (72% without shared influence). The influence of maternal genes seems to be predominant in birds (Asghar *et al.* 2015; Horn *et al.* 2011; Reichert *et al.* 2015, but Bauch *et al.* 2019; Viblanc *et al.* 2020) but contrasted and dependent on the offspring sex in sand lizards (Olsson *et al.* 2011). An extensive meta-analysis in humans has shown that the genetic inheritance of telomeres could not, without further studies, be more attributed to mothers than fathers (Eisenberg 2014). In the other hand, the paternal age at conception is now a well-established factor in humans, positively associated with telomeres: the older the father is, the longer his offspring's telomeres (Broer *et al.* 2013; Aviv 2018; Eisenberg and Kuzawa 2018; Horvath *et al.* 2019; Eisenberg *et al.* 2019). Such a phenomenon has been little studied in other species (reviewed in Eisenberg 2019) compared to what has been done in humans, but the results are very contrasted. For example, the relationship between paternal age at conception and offspring's telomere length is positive in sand lizards (Olsson *et al.* 2011), non-significant in Soay sheep (Froy *et al.* 2017), and even negative in jackdaws (Bauch *et al.* 2019). The parental genetic and epigenetic influences therefore remain to be fully explored, notably by integrating less studied species of various taxa. Although partially inherited (see Dugdale and Richardson 2018 for a critical review of telomere heritability), telomere length is also strongly influenced by the social and ecological environment (Hall *et al.* 2004; Epel *et al.* 2004; Geiger *et*

*al.* 2012; Stier *et al.* 2014; Nettle *et al.* 2015; Salmón *et al.* 2016; Angelier *et al.* 2017; Spurgin *et al.* 2017; Hammers *et al.* 2019; Viblanc *et al.* 2020). Furthermore, telomeres also reflect the energy trade-offs that animals make over the course of their life history. For example, it has been shown that individuals forced to develop more rapidly, and thus neglecting somatic maintenance in favour of growth, have shorter telomeres (Ringsby *et al.* 2015; Vedder *et al.* 2017; Monaghan and Ozanne 2018). Besides, guanine triplets found in vertebrate telomere sequences are a preferred target for oxidative molecules (Kawanishi and Oikawa 2004), explaining the shortening of telomeres under oxidative stress conditions, including *in vivo* studies (reviewed in Reichert and Stier 2017; but Boonekamp *et al.* 2017). Hence, telomeres appear to be an important element in the study of ageing since they integrate both the genetic and environmental components and reflect the compromises of life-history traits. In birds and mammals, there is mounting evidence that the shortening rate of telomeres is a reliable predictor of individual longevity, much better than chronological age (Pauliny *et al.* 2006; Bize *et al.* 2009; e.g. Barrett *et al.* 2013), as well as of the interspecific differences in longevity (Tricola *et al.* 2018; Whittemore *et al.* 2019). The question then arises, whether lengthening telomeres could increase life expectancy. To test this hypothesis several experiments have been attempted using telomerase, which is an enzyme capable of lengthening the missing end of telomeres after replication (Blackburn 2005). Its activation via TA-65, a molecule extracted from the root of *Astragalus membranaceus*, lengthens telomeres and have increased the healthy life span (termed as healthspan) in adult mice in the laboratory (Jesus *et al.* 2011) and improved several indicators of somatic status and good health in female volunteers (Harley *et al.* 2010). Birds treated with TA-65 have been found to have extended telomeres and a faster rate of feather regeneration (Reichert *et al.* 2014a). Although these and other studies (reviewed in Boccardi and Paolisso 2014) highlight the potential benefits of telomerase for healthspan extension, it should be noted that telomerase inhibition in somatic cells is believed to be a mechanism preventing uncontrolled cell division and thus the development of cancers (Harley *et al.* 1994; Seluanov *et al.* 2007; Gorbunova and Seluanov 2009; Artandi and DePinho 2010; Shay and Wright 2011). Nevertheless, lobsters (Klapper *et al.* 1998) or some long-lived birds (Haussmann *et al.* 2007) maintain telomerase activity in somatic tissues throughout their life without the development of multiple cancers. These heterogeneous results suggest that extending longevity by slowing down telomere attrition requires further studies to better understand cellular processes and thus prevent the appearance of possible adverse effects. Furthermore, a meta-analysis of 27 studies in non-avian mammals, birds and reptiles (Wilbourn *et al.* 2018) shows that although the general trend is towards a positive association between long telomeres and longevity, for some species there is no significant association or the association is even reversed (one study, Ujvari and Madsen 2009). Furthermore, in the *L. niger* ant neither telomerase activity nor telomere length apparently reflects the difference in longevity between queens and workers (Jemielity *et al.* 2007).

We have seen above ageing mechanisms related to damage to biological macromolecules (*e.g.* proteins, lipids, DNA) over the lifetime of an individual, with particular emphasis on telomeres, which appear to be a very conserved mechanism with strong explanatory power, at least in mammals and birds. However, telomeres seem not to explain so far the entire part of the variation in lifespan both at the interspecific or inter-individual levels. Below, I present additional mechanisms that are inherent to the very functioning of cellular metabolism and related to nutrition (see **Figure 2** for a schematic overview).

As illustrated in the introduction, it has been the intuition of mankind for many centuries that a sober diet is a key to longevity. However, it is only recently that the underlying mechanisms have begun to be deciphered. Studies in genetic model animals (*C. elegans* and *D. melanogaster*) have shown that calorie restriction and genetic manipulation are more effective than medication in increasing healthy longevity. Besides, analysis of genetic manipulations revealed that the signalling pathways that most increase healthspan are associated with calorie restriction (Liang *et al.* 2018). Calorie restriction, which is a type of dietary restriction, slows ageing in rodents (Simons *et al.* 2013), improves memory in older humans (Witte *et al.* 2009), and delays the onset of disease and death in rhesus monkeys (Colman *et al.* 2009). The phenotype associated with calorie restriction shows a profound change in metabolism by promoting the use of lipids as an energy source, decreasing mitochondrial activity but increasing the amount of circulating adiponectin and inducing increased sensitivity to insulin via the insulin / insulin-like growth factor (insulin/IGF) signalling pathway (mechanisms reviewed in Anderson and Weindruch 2010). These changes are probably regulated by at least two interconnected metabolic pathways involved in calorie restriction: the pathway of histone acetylases called sirtuins, notably sirtuins 1 to 3 (Cohen *et al.* 2004; Guarente and Picard 2005; Boily *et al.* 2008; Someya *et al.* 2010; Chen *et al.* 2018) and the mTOR pathway (mechanistic target of rapamycin, Gwinn *et al.* 2008; Jiang *et al.* 2008). These two pathways notably activate the transcription co-factor PGC1$\alpha$ which induces reprogramming of mitochondrial function under calorie restriction (Anderson and Weindruch 2010; Anderson and Prolla 2009 for further details about PGC1$\alpha$). This change in mitochondrial metabolism could explain the lower production of ROS and the decrease in the number of mitochondrial DNA mutations observed under calorie restriction (Barja 2002, 2004). Furthermore, both the mTOR pathway (Blagosklonny 2008; Pu *et al.* 2017; Li *et al.* 2018; Liu *et al.* 2018) and sirtuins (Guarente and Picard 2005; Morselli *et al.* 2010) activate autophagy which ensures the renewal of cellular organelles and has been associated with greater longevity and slower ageing (Cuervo *et al.* 2005; Bergamini *et al.* 2007; Morselli *et al.* 2010). To date, the only experiments that have undoubtedly increased longevity in several taxa have involved calorie restriction directly (Lin *et al.* 2001; Heilbronn and Ravussin 2003; Rogina and Helfand 2004; Greer *et al.* 2007) or indirectly via the mTOR pathway (Harrison *et al.* 2009; Wilkinson *et al.* 2012). This is a strong

argument to investigate these metabolic pathways in comparative studies of ageing. However, some authors, based on the stability-longevity hypothesis, predict that calorie restriction will beneficiate species with high pace of life (*i.e.* early sexual maturity, large litter size, narrow reproductive span) but hardly extend life span of species with low pace of life, such as humans (Demetrius 2004).

**Box: Which proximal mechanisms for which evolutionary theory of ageing?** This box aims at linking the previous two figures and highlighting the compatibility between theories and observed cellular mechanisms. As stated in **Figure 1** and main text, the ***mutation accumulation*** theory explains that extrinsic mortality, including predation, selects genes coding for traits related to reproduction or short-term survival, at the expense of genes coding for somatic maintenance. In addition, the ***disposable soma*** theory, which shows that, because of limited resources, trade-offs made during lifetime may result in a reduction in life expectancy in favour of a temporary need: e.g. immunity, reproduction. These two theories explain how oxidative damages accumulate and telomere erode over the course of an individual's life. On the other hand, the mTOR pathway (**Figure 2**) is necessary during embryonic development in *Mus musculus* (Murakami et al. 2004). In the same species, its harmful involvement in senescent mechanisms has been clinically demonstrated, since its inhibition extends life span by up to 14% in females and 9% in males (Harrisson et al. 2009). More generally, a higher metabolism and energy availability are often necessary in the early stages of life, but prove to be detrimental thereafter. This is exactly what the ***antagonistic pleiotropy*** theory describes. It is therefore particularly suitable to understand the mechanisms related to cell metabolism and energy status. Furthermore, the Nox enzymes use ROS as essential signal molecules in the innate immune response or in thyroid hormone synthesis, but they are also involved in the development of Alzheimer's disease or hypertension in elderly. Antagonistic pleiotropy can therefore also in this case explain that no mechanism has evolved to get fully rid of ROS, despite their potential detrimental effects. Under the hypothesis of an active and programmed selection of ageing (**Figure 1**, ***altruistic ageing***), genes involved in mechanisms generating oxidative stress could be actively selected for their late deleterious effects. Similarly, such pro-ageing genes could, for example, promote high metabolism, activate the mTOR pathway but inhibit the sirtuin pathway.

In summary, the mechanisms involved in the accumulation of damage in an organism during its lifetime (oxidative stress, telomere attrition) are believed to have been co-selected with life-history traits, and then when measured at one life stage, to predict the survival chances of classical model organisms (mainly mammals and birds). However, some organisms seem to escape the predictions made by these ageing models and clinical tests do not systematically give the expected results. It therefore appears that the accumulation of damage, although reflecting part of the biological reality of ageing, do not explain its whole complexity. Other mechanisms should be at work that complement or control them. More recently, other processes related to the functioning of cellular metabolism and energy availability have been underlined in human clinical trials. These mechanisms, joined in the hyperfunction theory (e.g. Blagosklonny 2013), define ageing not as a consequence of damage accumulation, but rather of the functioning of the cell itself. Although promising clinical results have been obtained, the full range of signalling pathways and their interactions have not yet been studied in detail and still need to be tested among a wider range of taxa. Ageing, mediated by the above mechanisms, occurs during life as a result of internal processes and the individual life history (*e.g.* chronological age, reproductive success,

immune response efficiency, growth rate), but always in interaction with the environment that modulates these internal processes. Especially, the significance of the social environment[5] has long been recognised in humans (*e.g.* Holt-Lunstad *et al.* 2010; Yang *et al.* 2016), but studies in animals are booming (reviewed in Sueur *et al.*, *unpublished*, see **Appendix 1**). For instance, social isolation may lead to seriously damaged health (Aydinonat *et al.* 2014; Koto *et al.* 2015; Dawson *et al.* 2018). There is also strong and repeated evidence linking social relationships to various disease-related outcomes, but the mechanisms that explain these associations remain largely unknown.

## Role of the social context in ageing processes

### *Evolutionary interconnections between sociality and longevity*

Animal species show varied social organisations that can be grouped according to their degree of complexity. First, in *solitary* species, contacts between individuals only take place at the time of reproduction, without any care for the young (*e.g.* solitary bees). *Gregarious* species do not have real social interactions but tend to gather in the same place and synchronize their activities (e.g. cockroaches Ame *et al.* 2004). From the stage of so-called *sub-social species*, care of the young is observed, even if sometimes rudimentary (e.g. dung beetle, Sato 1997). *Colonial* species form large colonies with a common rearing area for the young but the parents only care for their offspring (*e.g.* penguins). In contrast to *communal* species where several adults cooperate in rearing young even if they are not their own (system present in insects, birds and mammals, Lukas and Clutton-Brock 2012 and references therein). Finally, the highest degree of sociality is *eusociality*. According to Wilson and Hölldobler (Wilson and Hölldobler 2005), three criteria must be met for a species to be qualified as eusocial: overlap of at least two generations, cooperation in caring for the young, and specialisation of individuals (*i.e.* distinct tasks are performed by distinct individuals, including reproduction). Although a broader definition may include humans (Foster and Ratnieks 2005), only two mammalian species are considered strictly eusocial: the naked mole-rat (*Heterocephalus glaber)* and Damaraland's mole-rat (*Cryptomys damarensis*) (Burda *et al.* 2000). Among invertebrates, some shrimp species are also described as eusocial (Duffy *et al.* 2000), but the group that includes the most eusocial species are the insects, termed as *social insects* (Wilson 1971 p. 19). Two major groups of social insects are known to date: termites belonging to the Blattoptera order, and bees, wasps and ants belonging to the Hymenoptera order.

[5] All interactions and relationships between individuals, generally of the same species, sharing the same habitat. It also encompasses the resulting processes such as access to information and emergence of cultures.

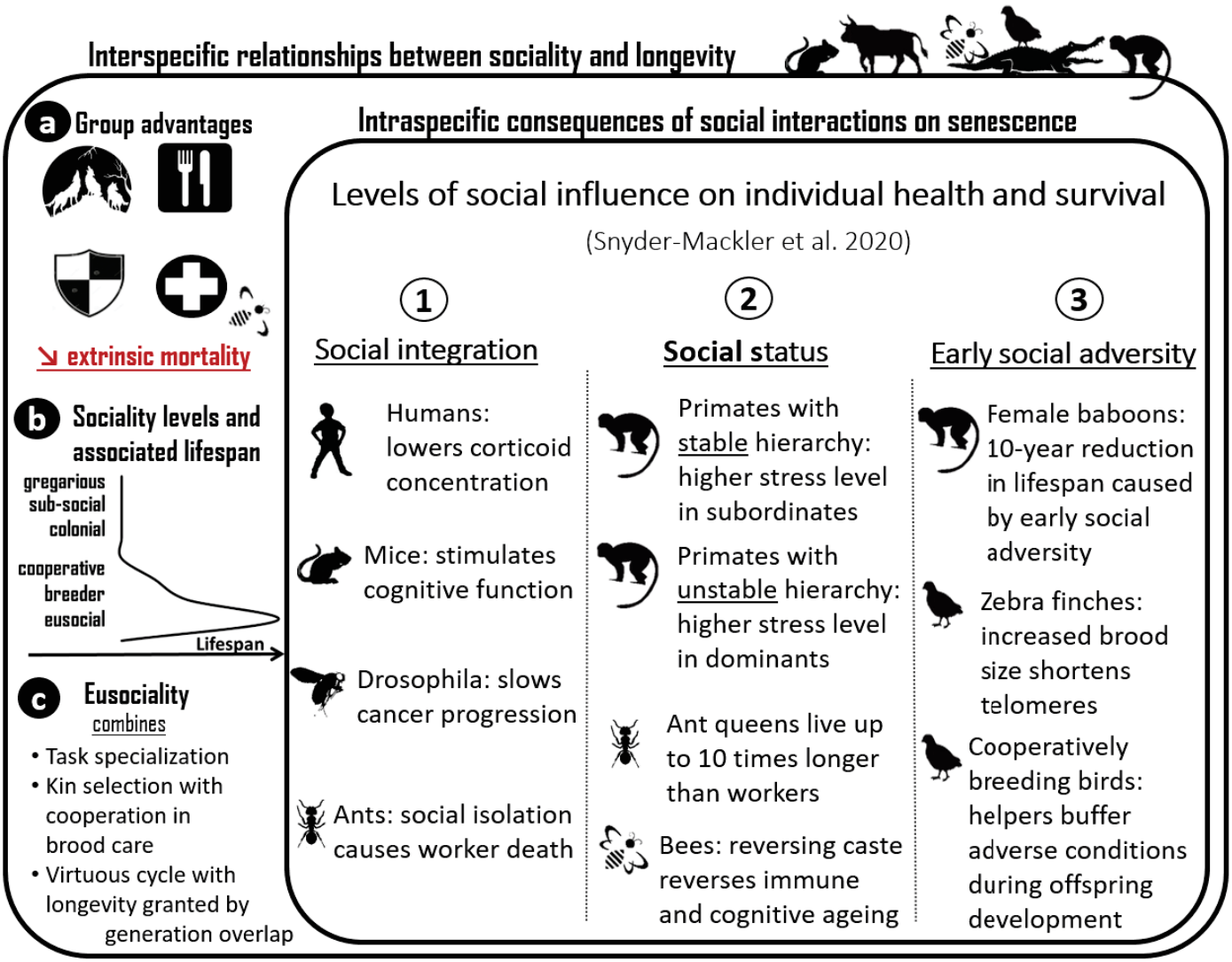


**Figure 3: Inter- and intra-specific associations between sociality and ageing.** Group living is known to provide several advantages **(a)** among them are: finding resources, territory and food defence, protection from predators, and social immunity in social insects. This contributes to decreasing extrinsic mortality and thus allowing longer lifespans to evolve. However, increasing the group complexity (sociality level) does not reflect in a linear increase in lifespan **(b)**. Only the eusocial way of life provide a doubtless gain in lifespan when compared to less social species. Studies in cooperative breeders (birds) are contradictory, and except a study in bovids (Bro-Jørgensen 2012) no less social taxa show an association between longevity and sociality. The exception of eusociality **(c)** is partly explained by the task specialization that frees the reproductive individuals from e.g. foraging or defence against predators, and allows them to invest the energy surplus into reproduction and somatic maintenance. Moreover, in eusocial colonies where individuals are highly inter-dependent because of task specialization and genetically close, the survival of an individual's relatives depends on its own survival, so the longer an individual lives, the more its inclusive fitness increases, fostering the emergence of longer lifespan. A long lifespan in turn incurs high social density, longer overlap between generations and more interaction with kin, reinforcing the evolution of cooperation. At the individual (intraspecific) scale, the sociality has various positive and negative consequences, according to the species ecology and behaviours. Social effects can be grouped into three categories: **(1)** social integration: initiating and maintaining positive interactions with conspecifics; **(2)** social status: individual situation that condition access to resources (e.g. food, mates, habitat); **(3)** early social adversity includes for example: maternal loss, high interspecific competition, a short gap until the birth of a younger sibling.

*Please refer to the main text (section 'Role of the social context in ageing processes') for the related scientific publications and further details.*

Group living brings immediate benefits to the survival of individuals of species that adopt this way of life (**Figure 3**). The first is the reduction of predation pressure either through a passive dilution effect (the probability of an individual being attacked decreases with the size of its group) or through active defence against predators (Williams 1957; Healy *et al.* 2014). The second direct benefit of group living is the greater efficiency in finding and defending a resource (Alexander 1974; Wrangham 1980). Both effects contribute to a decrease in extrinsic mortality (Abrams 1993; Ricklefs 1998; Lemanski and Fefferman 2018). This makes possible the selection of mechanisms to repair cellular damage since individuals may reach a later age, when such mechanisms may become necessary. Eusocial species take one step further in interlinking sociality and longevity. The specialization of tasks frees breeding individuals from many functions that they no longer have to perform to ensure their survival: nest building, foraging, and even to some extent immunity, which is ensured by the entire colony (Cremer *et al.* 2007; Walker and Hughes 2009; Hamilton *et al.* 2011; Aanen 2018). In this way, energy surplus can be invested in reproduction and longevity. These effects of social organisation on longevity in eusocial species are reinforced by kin selection. Indeed, living in a group encourages interactions within kin (Bourke 2007; Pavard *et al.* 2007). The survival of an individual's relatives therefore depends on its own survival, so the longer an individual lives, the more its inclusive fitness increases, favouring the appearance of great longevity. Inclusive fitness includes not only individual success in passing genes but also the success of relatives. According to the rules of kin selection, the age at which an individual begins to increase inclusive fitness is related to the onset of senescence (Hamilton 1966; Sherman *et al.* 1991; Bourke 2007). Thus, non-breeding individuals who begin to help their relatives before sexual maturity age more quickly. In addition, these non-breeding individuals are still highly exposed to extrinsic mortality, particularly when they go to forage for food outside the colony. This may therefore partly explain the emergence in eusocial species of social castes with distinct longevities.

Above, we have seen three mechanisms that explain the theoretical evolutionary pressures of sociality on longevity: decrease in extrinsic mortality, kin selection and energy saving due to task specialization in eusocial species. However, as Lucas and Keller have pointed out, longevity can also influence sociality (Lucas and Keller 2020, Figure 1). Indeed, greater longevity induces greater social density, longer overlap between generations and more interaction with kin, promoting the evolution of cooperation within a species (Carey 2001; Ross *et al.* 2015). Thus, longevity and sociality are caught in a virtuous cycle (Carey and Judge 2001). However, this virtuous cycle seems to require a high level of sociality to provide significant benefits. Indeed, studies that demonstrate a positive effect of group size or the number of social relationships on survival are carried out on species with large population sizes (n = 10-100): female chacma baboons (Silk *et al.* 2010), bighorn sheep (Vander Wal *et al.* 2015), weaver birds (Brown 2003), humans (Holt-Lunstad *et al.* 2010) and insects (Miramontes and DeSouza 1996;

Mersch *et al.* 2013; Koto *et al.* 2015). On the contrary, this relationship is not present in species living in small groups (n ≤ 6), such as Seychelles Warblers (Brouwer *et al.* 2006), yellow-bellied marmots (Blumstein *et al.* 2018), Pallas's mastiff bats (Gager *et al.* 2016). In addition, interspecific studies (reviewed in Lucas and Keller 2020, Table S2) taking into account phylogeny have failed to show a relationship between group size and survival in both birds (Møller 2006) and mammals (Kamilar *et al.* 2010), except for one study in bovids (Bro-Jørgensen 2012). Rather than using group size as a sociality criterion, it is also possible to categorize species according to sociality levels (*e.g.* cooperatively breeding vs. solitary species). In this case, studies in birds are equivocal, with some showing that cooperative breeders live longer (Arnold and Owens 1998; Downing *et al.* 2015) and others not (Blumstein and Møller 2008; Beauchamp 2014). Conversely, studies in eusocial mammals and insects clearly show a positive effect of a high degree of sociality on longevity. Particularly in reproductive individuals, since for queens of social insects, this is equivalent to an increase in their lifespan of 100 times compared to solitary insect species (Keller and Genoud 1997). In the naked mole-rat, this increase in lifespan remains valid even when comparing non-reproductive individuals to other solitary rodents (Buffenstein 2008) and also taking into account the fossorial lifestyle (Healy 2015). A cautious hypothesis would be that the benefits of sociality on longevity require the evolution of a eusocial organisation to be significant and that such a social organisation benefits mostly to reproductive individuals. However, studies interested in disentangling the influences of longevity and sociality are still few and lack a standardised methodology, particularly concerning the criteria to measure longevity and degree of sociality (Lucas and Keller 2020). Furthermore, even if at the interspecific level the influence of sociality on longevity may appear to be restricted to certain taxonomic groups in the current state of our knowledge, we will see in the next section that at the intraspecific level, the social interactions play a major role in ageing processes among a wide variety of species (Sueur et al., *in review in Trends in Ecology and Evolution*, **appendix 1**).

*Individual consequences of social interactions on senescence*

At the intraspecific level, the role of sociality in the ageing process is analysed by trying to understand the influence of an individual's role in the social system of the species. Social determinants are among the strongest predictors of individual mortality and morbidity in human and non-human mammals (extensively reviewed in Snyder-Mackler *et al.* 2020), and even in bees where a metrics derived from the social interactions predicts individual survival 157 % better than age (Wild *et al.* 2020). In their review, Snyder-Mackler and collaborators have shown that this influence of social determinants is highly comparable across diverse taxa (shared evolutionary origin) and can be grouped into three categories, summarised in **Figure 3**:

- (i) *social integration*, *i.e.* the ability to initiate and maintain affiliative interactions. The simple absence of interaction with conspecifics, *i.e. social isolation*, can have dramatic consequences: cancer progression (Dawson *et al.* 2018) or a weaker oxidative resistance (Ruan and Wu 2008) in Drosophila, cognitive decline in mice (Boyer *et al.* 2019), telomere shortening in African grey parrot (Aydinonat *et al.* 2014), increase in corticoid concentration in cichlid fish (Hannes and Franck 1983), zebra finches (Banerjee and Adkins-Regan 2011) and social mammals including humans (Grant *et al.* 2009; Hawkley *et al.* 2012), and even causes death in ant workers (Koto *et al.* 2015). The survival likelihood of foals has been proved to be enhanced by the number of individuals directly interacting with them (Nuñez *et al.* 2015). Similarly, the more male killer whales are integrated into the social network of their group, the higher their likelihood to survive, particularly in case of food scarcity (Ellis *et al.* 2017). In non-human primates, an increase of social integration by maintaining strong and consistent social interaction extends the lifespan of baboons and macaques (Silk *et al.* 2010; Archie *et al.* 2014; Thompson and Cords 2018; Ellis *et al.* 2019; Campos *et al.* 2020). In wild Barbary macaques, it has been shown that aggressive interactions can also contribute to individual survival when facing extreme weather conditions, potentially through a stabilising effect of affiliative relationships (Lehmann *et al.* 2016).

- (ii) *social status*, *i.e.* the situation of an individual that conditions the access to resources (*e.g.* food, partners, social support). From studies of captive primates, we know that the rank induces the physiological profile, and not conversely because the subsequent rank cannot be inferred from the physiological profile before the animals are placed in social groups. Being at the top of the hierarchy can be a way to improve fitness through privileged access to food and/or sexual partners. For instance, in black-capped chickadees (Schubert *et al.* 2007), female rhesus macaques (Blomquist *et al.* 2011), and wild rabbits (Holst *et al.* 1999) higher-ranked individuals have a longer reproductive lifespan. However, being high-ranked can also be a source of oxidative stress in species with cooperative breeders or unstable ranks over time, due to the many conflicts related to the establishment and maintenance of the social status (Masataka *et al.* 1990; Cavigelli 1999; Creel 2001; Sands and Creel 2004; Wapstra *et al.* 2011; Beaulieu *et al.* 2014; Cram *et al.* 2015). In meerkats, telomeres of higher-ranked individuals shorten faster (Cram *et al.* 2018). As highlighted by Sapolsky (2005), this situation can be reversed in species with stable hierarchical relations overtime where subordinate individuals show higher stress levels than dominants (e.g. Barnett 1955; Eberhart *et al.* 1983). In eusocial species, each individual has a determined place in the social organisation of the colony: the caste. This determines the individual behavioural repertoire but may also contribute to individual differences in longevity. Indeed, breeding individuals can live up to 10 times longer than workers (Keller and Genoud 1997). Even in a protected environment, major female workers of the weaver ant *Oecophylla smaragdina* die faster than minor workers, indicating that

the rate of ageing is caste-dependent, even within workers (Chapuisat and Keller 2002). In the honeybee, the young workers perform tasks inside the nest, then, as they get older, they become foragers and bring back food from outside the nest. It is possible to force the old foragers to resume within-nest activities (Münch *et al.* 2013) and this operation restores the typical phenotype of young workers, especially regarding their cognitive capacities and immune system (Amdam *et al.* 2005; Münch *et al.* 2008; Baker *et al.* 2012).

- (iii) *social early life adversity*. Adverse social conditions include, for example, low social status, maternal social isolation, maternal loss, high interspecific resource competition, a short gap until the birth of a younger sibling, early-life drought. Female baboons that experienced three or more of these adverse conditions lived an average of 10 years less than those that experienced no more than one (Tung *et al.* 2016). A decrease in lifespan associated with transient rank acquisition has also been observed in spotted hyenas (Strauss *et al.* 2020). The presence of adult helpers in cooperative breeders alleviate the cost of harsh environmental conditions during early development of juveniles (Russell *et al.* 2002; Covas and du Plessis 2005; Covas *et al.* 2008). Besides, helpers also reduce the cost of breeding for parents, increasing the likelihood of successful additional reproduction and survival (e.g. Russell and Lummaa 2009). Cooperative breeders show us that social system, and not only the individual role, can have consequences on individual fitness.

## Aims and scopes

We have seen, on the one hand, that the social role of an individual (*e.g.* dominant in a primate species, worker in a social insect) undeniably impacts its own ageing mechanisms or those of conspecifics (*e.g.* conflicts for the hierarchy, lower reproductive costs thanks to helpers). However, we still lack interspecific studies including less-studied taxa to draw definitive conclusions on the potential increase in life expectancy granted by a higher sociality level to all the individuals of a species. As far as we currently know, only eusociality seems to provide non-controversial benefits in terms of longevity. Moreover, the cellular and molecular mechanisms that may account for such benefits are not yet clearly elucidated. These two questions are addressed by the present thesis, the main objective of which is to explore the influence of the social organisation on senescent mechanisms. The objective is twofold. First, we address the links between longevity and sociality - both at the species and individual levels. Since the proximal mechanisms at the origin of the variability in organisms ageing rate are still being discussed, our second objective is to test and explore how well-known ageing cellular mechanisms are conserved in different taxa in relation to their sociality. The presentation of our results is organised as follows: six chapters, each containing one article and sometimes additional explanatory

text. These six chapters are grouped into three main parts detailed below. The list of articles is available at the very beginning of the manuscript (see Table of contents) and the other articles written during the PhD are available as appendices at the end of the manuscript.

Depending on the nature and complexity of a species' social organisation, its influence can take different forms. **Part 1** encompasses two studies addressing how the social environment impacts the ageing rate in two bird species characterised by different degrees of sociality. Thus, **Chapter 1** focuses on the zebra finch (*Taeniopygia guttata*), a sub-social bird species. In this species organised following a low degree of sociality, the social context varies essentially according to the number of individuals that can interact. We therefore modified the social density in a controlled situation and observed the effects on the physiology of individuals, notably by measuring telomere length and oxidative balance. Then, **Chapter 2** looks at a more complex social organisation: cooperative breeding in the sociable weaver (*Philetairus socius*). This species builds very large communal nests with chambers in which lives a pair. Such nests can harbour more than a hundred pairs (White *et al.* 1975). Some young adults, called helpers, do not reproduce and assist the parents in rearing the chicks. Not all pairs benefit from this assistance. Thus, sociable weavers offer a unique opportunity to test the impact of cooperative breeding on the ageing rate in chicks within the same species. This social organisation almost entirely meets the three criteria of eusociality: cooperation in caring for offspring, the overlap of at least two generations, but the division of labour is only partial. Indeed, the helpers are not a sterile caste exclusively dedicated to feeding the offspring, since the parents participate in the rearing of the chicks and nothing prevents the helpers from reproducing in a subsequent year. The influence of helpers on the reproductive success and survival of the parents has already been investigated by many studies that tend to demonstrate an advantage of this mode of reproduction for the parents (Russell and Lummaa 2009; Kingma *et al.* 2010; Tanaka *et al.* 2018). On the other hand, the influence of the helpers on the offspring they help to raise is still little studied, particularly considering survival and longevity. The study presented in this Chapter 2 analyses the effect of the presence of helpers before and/or after hatching on telomere length and survival of chicks over six years. These first two chapters enable us to understand the influence of progressive complex sociality on ageing in classical animal models (birds) and via biomarkers of ageing that are well established in this taxonomic group (oxidative balance and telomere length).

After the study of those sub-social and cooperatively breeding bird species, the next step in the study of the complexity of social organisation and ageing is the study of eusocial species. True eusociality is only found in very few taxa and mainly in hymenopteran insects. Several studies have shown that the mechanisms usually associated with ageing do not always seem to match observations in social insects (termites, bees, ants, wasps), appointing them as good candidates for finding new

ageing mechanisms of importance that may uncover ageing – sociality still undefined interactions. Thus, because of their high level of sociality and the exceptions they provide to the classical theories of ageing, social insects fit perfectly with the objectives of my PhD (Parker 2010). **Part 2** (1 chapter) and **Part 3** (3 chapters) tackle this opportunity in ants (Formicidae family). Worker and queen ants differ both in longevity and behaviour. Long-lived queens are restricted to the reproductive function of the colony, whereas workers are divided into several castes with very diverse tasks: *e.g.* feeding of larvae, food supply, corpse removal (Robinson 1992). As mentioned above, studies on social insects and ageing seem to indicate that classical mechanisms (*e.g.* telomere length, oxidative balance, reproduction/longevity trade-off) do not seem to operate as expected based on vertebrates ageing knowledge. However, these studies take into account only a small part of the diversity of social insect species, and the division of labour that characterises a eusocial species can be seen in multiple variations among the nearly 15 000 species of ants known to date (Bolton *et al.* 2006). **Chapter 3** tests whether classical biomarkers of ageing (telomeres, telomerase, and oxidative status) are decoupled from lifespan at the interspecific level, regardless of their sociality. To this end, we have studied the differences in terms of ageing markers between queens and workers in several species over a gradient of social organisations, sometimes close to the limit of no longer being eusocial. The ageing markers chosen are oxidative balance, telomere length and telomerase activity. While Chapter 3 tests the accuracy of classical mechanisms through a multi-species analysis, the last three chapters, grouped in **Part 3**, aim to highlight ageing processes little or not explored so far but at work in social insects. For this purpose, I have grouped in this last part three exploratory proteomics and metabolomics studies (**Chapters 4**, **5** and **6)** in one eusocial insect species: the black garden ant (*L. niger*). These studies benefit from using proteomics and metabolomics, rather than genomics or transcriptomics, to jointly explore the influences of social organisation on ageing. Moreover, unlike most past studies, they not only compare queens and workers, but also worker castes to each other. The mechanisms highlighted by the present work opens new avenues addressing the question of the coevolution of ageing with sociality, that hopefully will be picked up in future studies in other taxa and thus complement our present understanding of the evolution of ageing mechanisms in general.

In summary, while the first part proposes to study classical mechanisms in birds, an animal model usually used in ecophysiology, the second part tests these same mechanisms but in social insects which seem to be an "ageing exception". Finally, the last part seeks to unravel hidden mechanisms that may participate in the evolution of the so peculiar ageing diversity of eusocial insects. Each chapter presented above is linked to an article that has been published, is being reviewed or is in the process of writing. When needed, a short introductory text accompanies it to facilitate the understanding of the place of the

article in the overall context of this PhD thesis. The details of the scientific context and methodology for each part are therefore stated before the article or in the article itself. At the end of the thesis manuscript, articles published during the thesis but not directly related to the co-evolution of sociality and ageing are presented as appendices.

# - First part -

# Examples of the social environment influence in two social bird species

# Chapter 1| Domino effect of conspecific aggression on oxidative status and telomere length in zebra finch

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Le ciel d'automne
Des milliers de moineaux
Le bruit de leurs ailes.

Autumn sky
Thousands of sparrows -
The sound of their wings.

Yotsuya Ryu

**Abstract**

Living in social groups may exacerbate inter-individual competition for territory, food and mates, leading to social stress with downstream consequences on individual physiology and health. Studies investigating the consequences of social aggression on individual stress have largely focused on glucocorticoid (so-called stress) hormones and highlighted that social stress may be incurred both by socially low- and high-ranking individuals, as a result of either being maintained at the bottom, or fighting to stay on top, of the social hierarchy. Fewer studies have investigating the cascading effects of social aggression on individual oxidative stress physiology, while considering both the amount of aggression emitted and received by individuals, that is central in defining social hierarchies. Here, we experimentally evaluated the cascading effects of social aggression on individual oxidative stress and cellular ageing in captive zebra finches (*Taeniopygia guttata*). Over a 64-day period of social interactions, we compared the effects of social aggression (received or emitted) on individual oxidative status (oxidative damage and antioxidant defences) and body condition, and downstream effects on relative telomere length attrition of birds living in low (~6 birds/$m^3$) vs. high (~12 birds/$m^3$) social density condition. At high social density, birds were more aggressive and increased their consumption of antioxidant-rich seeds (red millet) as a proportion of other seeds in their diet. Using pathway analysis, we found that the more aggression a bird received over the experiment, the higher its oxidative stress response: it incurred higher oxidative damage (*i.e.* higher plasma levels of Reactive Oxygen Metabolites, and especially of 8-oxo-deoxyguanosine a marker of DNA damage), and showed activation of antioxidant defences (increased antioxidant capacity of the plasma, OXY-adsorbent test). In turn, this oxidative stress response induced a greater telomere attrition between the beginning and the end of the experiment. Further, received aggression also had a direct negative effect on telomere dynamics that were not related to oxidative stress. In contrast, the amount of aggression emitted by an individual had no significant effect on individual oxidative stress or telomere attrition. Aggression did not affect bird body condition over the course of the experiment, nor were changes in body condition associated with changes in telomere length. Our study sheds light on the causal chain from social interactions to oxidative balance to telomere attrition. The long-term behavioural or physiological responses and consequences of socially induced stress remain to be characterised.

**Keywords:** social environment, competition, behavioural ecology, oxidative stress, path analysis, *Taeniopygia guttata*

## 1. Introduction

From anti-predation benefits (Hamilton 1971), through the sharing of information between conspecifics (Murton 1971), facilitated access to mating partners (Clutton-Brock *et al.* 2001), to cooperation between group members (Deneubourg *et al.* 2002; Sachs 2006; Clutton-Brock 2009), the advantages of group-living are numerous. By the same token, group-living also incurs a certain number of costs such as increased transmission rates of parasites and pathogens (Côté and Poulinb 1995; Manlove *et al.* 2014), the potential for cuckoldry (Hoogland and Foltz 1982; Philipp and Gross 1994; Roux *et al.* 2013), the necessity to avoid inbreeding through dispersal with associated risks (Lukas and Clutton-Brock 2011), or competition for foraging, territorial, reproductive, or other resources (Craig 1921; Wong and Balshine 2011).

Although the individual fitness benefits of group-living must outweigh its costs for sociality to evolve, group-living animals are faced with the number one challenge of balancing individual needs with that of other group members (Jones 1980; Buss 1981; Shrader *et al.* 2007). Conflicts over resources are often resolved by aggression (Aureli et al. 2002), or by the establishment of dominance hierarchies. Both may lead to individuals being injured or even killed (Jones 1980; Hof and Hazlett 2012). It is worth noting that the lack of social interactions – or social isolation – may also have deleterious consequences on individual health and fitness, *e.g.* in fishes (Hannes and Franck 1983), insects (Koto *et al.* 2015), birds (Apfelbeck and Raess 2008; Banerjee and Adkins-Regan 2011; Aydinonat *et al.* 2014) and mammals (Harlow *et al.* 1965; Hawkley *et al.* 2012). Thus, group living (or lack thereof) can present a potent source of stress for social animals.

In vertebrates, the consequences of social environments on individual stress have traditionally been assessed by examinations into the functioning of hypothalamic-pituitary-adrenal (HPA) axis in response to variation in social contexts or group composition (Sapolsky 1983; Denver 1999; Yao and Denver 2007). For instance, we now understand that in social groups, both high- and low-ranking individuals may experience substantially elevated levels of stress hormones (glucocorticoids, see below), as a result of either being maintained at the bottom, or fighting to stay on top, of the social hierarchy (Fox *et al.* 1997b; Creel 2001; Goymann and Wingfield 2004; Sapolsky 2005). However, the study of glucocorticoid variation alone only provides a partial picture, since both short-term and long-term increases in glucocorticoid hormones may have varying positive or negative consequences on individual health and fitness (Gormally *et al.* 2019), including potentially positive trans-generational consequences (Evans *et al.* 2006; Dantzer *et al.* 2013; Zimmer *et al.* 2013). One alternative and complementary framework, is to focus on downstream measures of individual stress, such as the ability

of socially-stressed individuals to maintain homeostasis, which may be reflected in impaired immunity, oxidative imbalance and the onset of oxidative stress, or damage to essential biomolecules such as DNA (Breuner *et al.* 2013; Gormally *et al.* 2019).

An individual's oxidative status in response to social environments is an especially interesting metric to consider for two main reasons. First, increased glucocorticoid levels have been positively related to increased oxidative stress among a broad diversity of vertebrates (Liu and Mori 1999; Costantini *et al.* 2011; Almeida *et al.* 2011). Oxidative stress occurs when an individual's antioxidant defences are no longer sufficient to offset the synthesis of oxidizing molecules, including reactive oxygen species (ROS). Because ROS originate mostly from normal cellular respiration (Ott *et al.* 2007), their presence is ubiquitous in aerobic organisms, and besides cellular signalling roles, they are known to functionally damage important biomolecules such as lipids, proteins and DNA. Further, ROS have been suggested to play an important role in the process of ageing (Liu and Mori 1999; Betteridge 2000; Birben *et al.* 2012), notably through their deleterious action on specific DNA sequences known as telomeres (Saretzki and Zglinicki 2002; Houben *et al.* 2008). Telomeres are repetitive sequences located at the ends of the eukaryotic linear chromosomes, and whose role is to protect the degradation of DNA coding sequences during successive cell divisions (Venkatesan *et al.* 2017). Because of their sensibility to stress, they have been proved to properly reflect harsh ecological or social environments (Epel *et al.* 2004; Nettle *et al.* 2015; Salmón *et al.* 2016; Spurgin *et al.* 2017; Hammers *et al.* 2019). Several studies across bird and mammalian taxa have shown that telomere dynamics predict long term survival better than chronological age (e.g. Bize *et al.* 2009; Whittemore *et al.* 2019). Second, several studies have recently highlighted both positive and negative relationships between social contexts and individual oxidative status on the one hand (Miyashita *et al.* 2006; Nation *et al.* 2008; Hargitai *et al.* 2009; Jiang *et al.* 2013; Cram *et al.* 2015; Lardy *et al.* 2016), and social contexts and telomere length on the other hand (Epel *et al.* 2004; Cherkas *et al.* 2006; Lansdorp 2006; Kotrschal *et al.* 2007; Aydinonat *et al.* 2014; Uchino *et al.* 2015; Lewin *et al.* 2015; Oliveira *et al.* 2016; Cram *et al.* 2017). However, the direct and indirect pathways linking social environments to telomere length through oxidative stress have seldom been addressed and results remained equivocal (Nettle *et al.* 2015; Nettle and Bateson 2017).

Here, we investigated the effects of aggression (emitted or received) on relative telomere length (rTL) in colonial zebra finches (*Taeniopygia guttata*). Zebra finches are highly social granivorous passerines that can live in groups of 50 to 100 individuals in which a social hierarchy is established. They are territorial both in wild and captive conditions (Evans 1970; Yamahachi *et al.* 2017), making them a good model system for studying the effects of social aggression on individual physiology. We

used a pathway analysis (Li 1975) to test for the direct and indirect effects of aggression on rTL, notably via cascading effects on individual oxidative stress. We recorded the aggressive behaviour of 36 zebra finches for 64 days, and assessed their oxidative status, rTL and body condition at the start and end of the experiment. Oxidative damage was assessed in plasma by measuring both an overall marker of oxidative damage (*i.e.* Reactive Oxygen Metabolites; d-ROM test; Costantini 2016), as well as a specific marker of damage to DNA (*i.e.* 8-Oxo-2'-deoxyguanosine; 8-oxo-dG). We also obtained a marker circulating antioxidant capacity using the OXY-adsorbent test (Costantini 2011).

In zebra finches, as in other species, social dominance is established by a set of specific beahaviours (Bonoan *et al.* 2013) influenced by many intrinsic factors, such as personality (David *et al.* 2011) or hormonal concentrations (*e.g.* testosterone; Ardia *et al.* 2010). In line with the social stress hypothesis, we expected birds at the bottom, or at the top, of the social hierarchy to suffer from chronic social stress either as a result of being subordinates, or fighting to stay on top, of the social hierarchy (Fox *et al.* 1997b; Creel 2001; Goymann and Wingfield 2004; Sapolsky 2005). Consequently, we expected birds that received or emitted more aggressions over the course of the experiment to suffer from chronic oxidative stress. This oxidative wave is expected to trigger an antioxidant response from the organism, and we therefore expected to observe both an increase in oxidative damage markers (reflecting oxidative stress), and an increase in plasma antioxidants (reflecting the response to oxidative stress). In addition, we expected those birds to suffer from greater telomere loss over the course of the experiment, since telomeres are highly sensitive to oxidative stress (Kawanishi and Oikawa 2004; but see Boonekamp *et al.* 2017). Furthermore, the birds that display the highest number of aggressive behaviours (*e.g.* pecking, chasing) are also those that receive the fewest, and conversely, those that receive the most are those that give the fewest (Bonoan *et al.* 2013). We therefore expect the aggression emitted to have opposite relationships to the aggression received: negative with markers of oxidative stress and positive with rTL. Finally, chronic stress should be negatively associated with body condition, which in turn is positively associated with long telomeres in birds (Le Vaillant *et al.* 2015; Criscuolo *et al.* 2018; Angelier *et al.* 2019). We thus predict a negative correlation between aggression and body condition, but a positive correlation between body condition and telomeres. By combining physiological and behavioural measures, rarely investigated at the same time, our study offers a broad picture of how social environment, behaviour, physiology, and ageing might interact.

## 2. Materials and Methods

### *2.1. Study birds and housing*

All birds in the study were individuals of reproductive age (between 1 and 3 years old). Each bird was ringed for identification. The study consisted in comparing the physiology and behaviour of birds

depending on the aggression they emitted and received. Since zebra finches are known to be more aggressive in denser social environments (Poot *et al.* 2012), we increased the natural variation in bird aggressive behaviour by housing birds either in low (N = 6 birds; 2 aviaries) or high (12 birds; 2 aviaries) social density aviaries. Each aviary was built to identical dimensions (150 x 100 x 72 cm) and comprised of 6 feeding perches, 2 resting perches and 3 nest boxes each (see Figure 1). On day one of the experiment, males and females were randomly assigned to an aviary, each kept in separate but identical rooms. The climatic conditions were set at a temperature of 24°C, 40% hygrometry and a 14:10 light-dark cycle. Water and food were provided *ad libitum*. Birds were fed with red millet *(Panicum miliaceum)*, yellow millet (*Panicum miliaceum*) and yellow panicum (*Setaria italic*) in equal proportion.

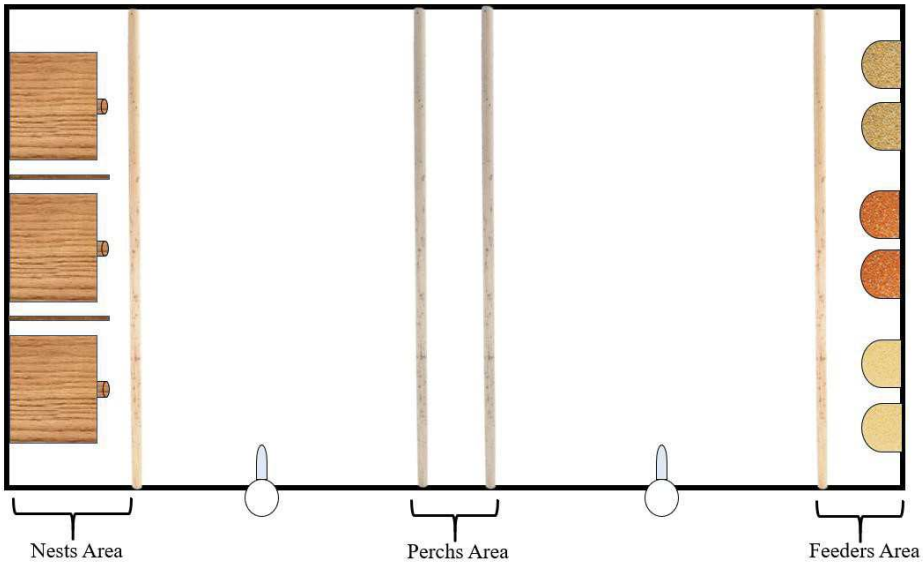


**Figure 1: Cage organisation from an upper view.** A cage measures 150x100x72 cm and is composed of three distinct areas. On one extremity, three squared wooden nest boxes at the same height, with one entrance and a rod in front of it, were placed and separated with 15 cm large wooden plates. On the opposite side and at the same height, we placed three pairs of feeders, containing seeds. In the cage's width, we put four identical perches at 37 cm from the roof: one in front of the nests, one in front of the feeders and two in the middle (10 cm apart from each other). Two water points are placed on one side of the cage, at equidistance from the three areas.

*2.2. Behavioural and physiological monitoring*

Bird behaviour was filmed every ten days for two hours using three GoPro Hero© (GoPro, Inc., USA) cameras per cage. Cameras were placed from above the aviaries, located 37 cm from the nests or perches, and set to film at a wide angle so that the entire width of the aviary could be seen on the videos. One camera was placed above the nests, one above the central perches and one above the feeders. The combination of the three cameras made it possible to record the whole aviary. We balanced filming hours between morning 08:30-12:30 and afternoon 12:30-18:30, to capture potential variation in bird activity during the day. Birds could be individually recognised by a small, numbered label paper tag

that was glued to a feather at the top of the head. On average, cameras recorded bird behaviour for 2.1 hours continuously (range: min = 1.9, max = 2.2). The slight variation between recording durations was due to differences in the battery capacity and specific models of the cameras.

On the first and final days of the experiment, we weighed birds on a precision scale (± 0.1 g) and took a blood sample of ca. 120 µL from the brachial vein using two 100 µL heparinized capillary tubes (see below). Bird structural size was measured (tarsus length, ± 0.1 mm) only once at the start of the experiment using digital callipers, since tarsus length does not change in adults (average length: 15.1 mm ± 0.8, min = 13.1 mm, max = 16.6 mm). The study was conducted over a total period of 64 days.

*2.3. Behaviour analysis*

Videos analyses were conducted using the Behavioural Observation Research Interactive Software (BORIS, v 7.4 Friard and Gamba 2016). We quantified the occurrence of aggressive behaviours emitted and received by specific individuals over the 2-hour recording periods, on each image frame of the video. The videos were scored by two separate observers with relatively high inter-observers repeatability (0.82, *i.e.* above 0.80 threshold typically used in behavioural studies; Hartmann, 1977; Watkins and Pacheco, 2000). Both observers watched the same number of videos. Aggressive behaviours included '*pecking*', *'chases'* and '*displacement*'. '*Pecking'* occurred when a bird emitted a single rapid or sequence of beak strikes directed towards another bird (beak, head, body, or tail). '*Chases*' occurred when a perched bird rapidly initiated a hoping movement towards another bird on the perch, forcing it to flee to another perch, without contact. '*Displacement*' occurred when a bird flew from a different perch to the location of a perched bird forcing it to flee to another perch with or without contact. In the analysis, a sequence of events was considered to be a single event (*i.e.* multiple fast bill hits were considered as *'pecking'*; we did not count every single peck).

*2.4. Effects of social density treatment on bird aggressive behaviour*

The first step was to control that higher density actually increase the number of aggressive social interaction. For this purpose, we used the frequency of agonistic interactions per bird and per hour of observation to determine if the high social density condition led to an increase in the rate of agonistic interactions compared to the low social density condition. As the number of aggressive behaviours per bird per hour was derived from count data, we performed a GLMM with a quasi-poisson distribution. We used group density and sex as independent variables and controlled for replicated treatments (2 high social densities, 2 low social densities) as a random factor.

*2.5. Consequences of aggression on individual physiology*

The second step was to assess the impact of aggression on telomere length both directly and indirectly through oxidative stress.

2.5.1. Oxidative stress and relative telomere length measurements

Blood samples (120 µL) were kept on crushed-ice during the time of sampling and centrifuged within the hour (10 min, 3500 rpm, RT) to separate blood cells – used for telomere measurements – from plasma – used for oxidative stress measurements. Samples were immediately stored at -80°C, until analysis. We assessed bird's oxidative status *via* longitudinal (at the start and end of the study) global measures of antioxidant defences and oxidative damage in blood plasma. Specifically, oxidative stress and non-enzymatic antioxidant defences in plasma were measured through colorimetric assays, using an infinite M200 microplate reader (Tecan Group Ltd. Männedorf, Switzerland). All those measurements were performed in duplicate and we controlled for interplate variation with a duplicated point repeated on each plate containing a goose plasma standard. Some blood samples were of a too little volume to allow all measurements to be taken or to re-analyse outlier data points. This leads to the presence of NA values (26/296 = 8.78%).

Antioxidant capacity (Costantini 2011) of bird's plasma was assessed using the OXY-Adsorbent test (DIACRON Labs, Grosseto, Italy) . This test measures the ability of antioxidant defences to buffer the action of the highly oxidative hypochlorous acid (HOCl). We used 5µL of 1:100 diluted plasma and measures are expressed as µM of HOCl neutralised per ml. The absorbance, measured at 505 nm, decreases when the antioxidant concentration increases. For the Oxy-Adsorbent assays, mean intraplate coefficient of variation was of 7.3 % and mean interplate coefficient of variation was of 8.2 %. Oxidative damage in birds' plasma was measured using the d-ROM test (DIACRON Labs, Grosseto, Italy) that measures the concentration of Reactive Oxygen Metabolites (ROMs, see (Costantini 2016). This test utilizes the organic molecules oxidised by free radicals in a chain oxidative reaction, the final oxidised molecule of which is a chromogen, which turns pink when oxidised. The more the sample contains free radicals, the higher the absorbance (505 nm). We used 4 µL of non-diluted plasma and measures are expressed in mg $H_2O_2$.$dL^{-1}$. For the dROM assays, mean intra- and inter-plate coefficient of variation was respectively of 7.5 % and 7.9 %. In order to obtain an oxidative damage metric related to telomeres, we measured the plasmatic concentration of 8-Oxo-2'-deoxyguanosine (8-oxo-dG), which is an oxidised derivative of deoxyguanosine after DNA oxidation. We assessed 50 µL of 1:20 diluted plasma samples in 8-oxo-dG ($ng.mL^{-1}$) through the Damage DNA (8-oxo-dG) ELISA Kit (StressMarq Biosciences, Victoria, Canada) as previously described in birds (Stier *et al.* 2014). The absorbance (450 nm) is inversely proportional to 8-oxo-dG concentrations. For the 8-oxo-dG assays, mean intraplate coefficient of variation was of 8.3% and mean interplate coefficient of variation was of 6.7 %.

In birds, erythrocytes are nucleated and relative telomere length (rTL) has been shown in these cells to be correlated with rTL in other tissues (Reichert *et al.* 2013). The DNA was therefore extracted directly

from the red blood cells (DneasyBlood and Tissue kit, Qiagen). We measured rTL by quantitative real-time PCR (qPCR) based on a previously implemented protocol (Cawthon 2002; Criscuolo *et al.* 2009). The rTL is then calculated from the T/S ratio, where 'T' is the copy number of the telomeric sequence and 'S' is the copy number of the control sequence. As a control, we used the gene of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Smith et al., 2011). We amplified the GAPDH gene with the following forward and reverse primers: 5′-AACCAGCCAAGTACGATGATGACAT-3′ (GAPDH-F) and 5′-CCATCAGCAGCAGCAGCCGCCTTCA-3′ (GAPDH-R). Telomere primers were Tel1b (5′-CGGTTTGTTTGGTTTGGTTTGGTTTGGTTGTTGTTTGTTGTTGTTGTT-3′) and Tel2b (5′-GGCTTGCCTTACTTACTTACCCTTACTTACTTACTTACTTCCTTCCTT-3′). Amplification by qPCR was performed with 5 ng of DNA, 200 nmol.L-1 of primers (Tel1b/Tel2b or GAPDH-F/GAPDH-R) and 5 μL of Power SYBR Green PCR Master Mix (AppliedBiosystems) in a final volume of 10 μL. Telomere and GAPDH sequences were amplified on one 384 wells plates. The qPCR conditions for telomeres were 10 min at 95°C, then 30 cycles of 1 min at 56°C and 1 min at 95°C. The qPCR cycles for the GAPDH sequence were as follows: 10 min at 95°C, followed by 40 cycles of 1min at 60°C and 1min at 95°C. All samples were run in duplicate. Each plate contained a serial dilution (10, 5, 2.5, 2.5, 1.25 ng) of a reference individual's DNA. A reference curve was then drawn from these points to evaluate the amplification efficiency (mean efficiencies for telomere and GAPDH sequences were 100.8 and 99.9 %, respectively). We took into account the slight variation of efficiency between telomere and GAPDH amplifications by calculating the T/S ratio according to Pfaffl's recommendations (Pfaffl 2001): $[(1+E_{telomere})^{\Delta Ct_{telomere}}]/[(1+E_{GAPDH})^{\Delta Ct_{GAPDH}}]$. E' is the amplification efficiency and 'ΔCt' the difference in time required to reach the fluorescence detectability threshold between control and sample ($Ct_{control} - Ct_{sample}$). Mean intraplate coefficient of variation was 2.67 ± 0.24 % for the Ct values of the telomere assays and 0.43 ± 0.04 % for the Ct values of the GAPDH assays. Intraplate coefficient of variation was 13.61 ± 0.99 % for the relative T/S ratios. Following Nettle et al. (2019), we also calculate the Intra-class correlation (ICC) to assess repeatability of T/S ratio, which was of 0.902. We checked the absence of non-specific amplification with a well containing PCR-grade water instead of DNA and by drawing the melting curve (primer-dimer).

2.5.2. Statistical analysis of the direct and indirect effects of aggression

We used standardised partial regression analyses (*i.e.* path analysis; Li 1981) to explore the causal relationships between bird aggression, variation in oxidative status and telomere dynamics over the course of the experiment. We included in this analysis both direct effects of aggression (received and emitted) on rTL, and indirect effects of aggression through modulation of bird oxidative stress. In the path model, the time budget spent in aggression for each bird (N = 36 birds in total, 18 males and 18 females) was calculated as the number of aggressive events (separated in aggression received or

emitted) divided by the total recording time (hours) of the aviary over the entire experiment. Since some oxidative measures (ROM, OXY, 8-oxo-dG ) were missing due to low plasma volume (26/296 = 8.78% of data), we used an iterative PCA algorithm to estimate missing values ('missMDA' package v.1.14, Josse and Husson 2016). This method is preferred to classical completion of missing data by the group's mean, leading to underestimating the true variance within the population (Little and Rubin 2019). After data completion, we calculated the difference between the start and end of the experiment for each oxidative balance marker (ROM, OXY, 8-oxo-dG), correcting for potential regression to the mean effects (see Kelly and Price 2005; Verhulst *et al.* 2013), and aggregated these measures into 2 principal components of a PCA (FactoMineR package v.2.6, Lê *et al.* 2008), which were included in the path model. Body condition was calculated as the residuals of the regression between tarsus length and mass (r = 0.83, t = 1.99, p-value = 0.03) at the start and end of the experiment. As for oxidative stress markers, we calculated the change in body condition and rTL over the course of the experiment, correcting for potential regression to the mean effects, before inserting them in the path model. All data was standardised prior to analyses and we checked the independent variables for collinearity through variance inflation factors (VIFs). All statistical tests were performed in R v. 3.6. (R Core Team 2019) at an alpha threshold of 0.05. We used the 'ggplot2' package for graphical representations (Wickham 2016).

*2.6. Effects of the social density treatment on bird seed consumption and beak colouration*

The final step was to test the capacity of birds to buffer the oxidative stress response through a potential self-supplementation in dietary antioxidant. We tested how the high/low social density treatment affected seed consumption. Birds were provided with ad-libitum access to three different seed types differing in their antioxidant content: red millet (141.1 μmol eq trolox/g) > yellow millet (67.9 μmol/g) > yellow panicum (59.8 μmol/g). Seed consumption was expressed as the average consumption of a given seed type per individual per day (i.e. the total seed mass consumed per aviary for each seed type divided by the number of days between two weightings and the number of birds in the aviary). We then calculated seed type consumption as a proportion of overall seed consumption to determine average individual diets (*e.g.* the proportion of yellow millet consumed relative to yellow millet, red millet, and yellow panicum). We then investigated how social density (independent factor; high/low density), seed type (independent factor; yellow millet/red millet/yellow panicum) and the interaction between both factors affected seed consumption, controlling for replicated treatments (2 high social densities, 2 low social densities) as a random factor in the model. Data was analysed using a GLMM with a quasi-binomial distribution, recommended for proportion data (Thiele 2012; Shannon 2013).

## 3. Results

We remind that, unless otherwise specified, the results below concern the differences during the experiment for ROM, OXY, 8-oxo-dG and telomere length (final value - initial value). High values of these differences therefore reflect longer telomeres or high concentrations of oxidative status markers. Although we initially constituted mixed groups of males and females, few individuals actually paired over the course of the study, and none actually bred (although some pairs engaged in nest building).

### *3.1. Effects of the social density treatment on bird aggressive behaviour*

Within aviaries, bird aggressive behaviour was more closely related than between aviaries (repeatability of 46%, $p = 0.004$, CI95 = [0, 0.772]). In the high social density condition, the total number of aggressive behaviours expressed by birds was 2.2 folds higher than in the low social density condition (GLMM: $t = -3.79$, $p < 0.001$, see **Figure 2**). More precisely, both the number of 'pecking' (GLMM: $t = -3.25$, $p = 0.002$) and 'chases' (GLMM: $t = -2.59$, $p = 0.012$) increased in the high-density group, while the number of 'displacements' (GLMM: $t = -0.12$, $p = 0.905$) did not significantly change.

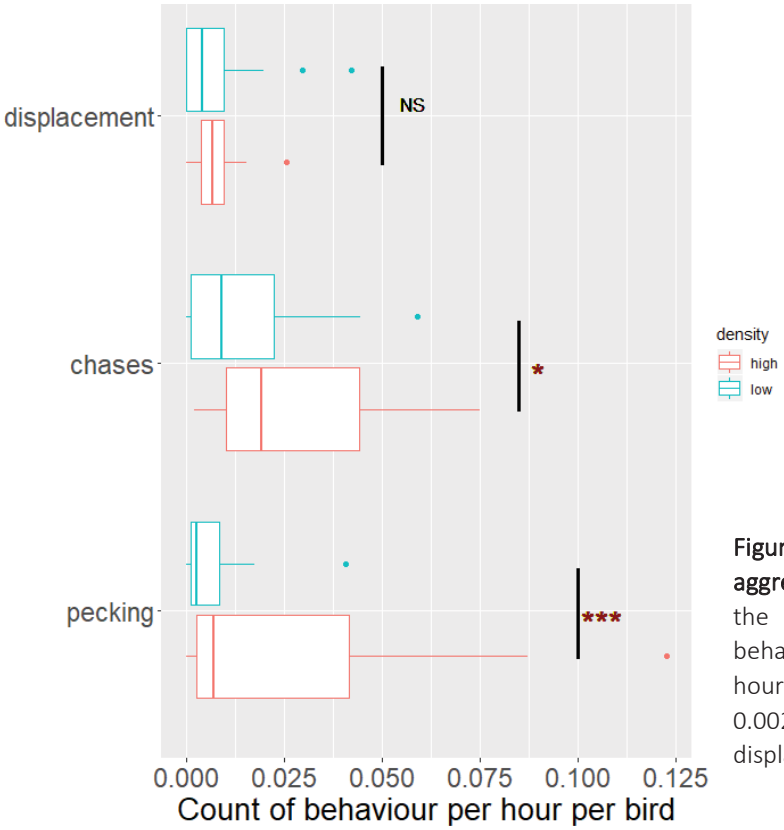


**Figure 2: Effects of social density on bird aggressive behaviour.** Boxplot showing the number of the three aggressive behaviours recorded in our study per hour per bird: pecking ($t = -3.25$, $p = 0.002$), chases ($t = -2.59$, $p = 0.012$) and displacement ($t = -0.12$, $p = 0.905$).

*3.2. Principal component analyses on oxidative markers*

The first, second and third principal components (PC 1-3) respectively explained 61.36%, 26.69%, and 11.95 % of the overall variance in ROM, OXY and 8-oxo-dG (**Table 1**). PC1 was strongly and positively correlated with all three variables (> 0.70) and accounted for 28.55% in ROM, 28.17% in OXY and 43.28% in 8-oxo-dG. PC2 was nearly independent of 8-oxo-dG but reflected an opposite association between ROM and OXY, with a respective coefficient of correlation of -0.63 and 0.64. We kept the first two most informative PCs for the subsequent path analysis, which accounted for over 85% of cumulative variance.

Table 1: Statistical values of the principal component analysis (PCA). For each variable are provided the coefficient of correlation to the given axis, followed (in brackets) by the contribution to the same axis. The contribution indicates what percentage of the variable makes up the axis in concern. The sum of the contributions on an axis is always 100%.

| | PC1 | PC2 | PC3 |
|---|---|---|---|
| **dROM** | 0.72 (28.55) | -0.63 (49.35) | 0.28 (22.10) |
| **OXY** | 0.72 (28.17) | 0.64 (50.65) | 0.28 (21.18) |
| **8-oxo-dG** | 0.89 (43.28) | -0.003 (0.00) | -0.45 (56.72) |
| **% variance** | 61.36 % | 26.69 % | 11.95 % |
| **Cumulated % variance** | 61.36 % | 88.05 % | 100 % |

3.3. Path analysis

The path model highlighted both direct and indirect effects of received aggression on changes in bird rTL over the course of the experiment (**Table 2 and Figure 3**). Received (but not emitted) aggression had a direct significant negative impact on rTL (-0.36, **Table 2**). Received aggression also had a significant indirect effect on rTL through modulation of bird oxidative status (indirect effect = -0.154; indirect paths are considered significant when both direct paths are as well, Cohen *et al.* 2013). Specifically, when received aggression increased, PC1 increased too (direct effect of 0.35) and induced a decrease in rTL over the course of the experiment (direct effect of PC1 on rTL = -0.44). All the other direct and indirect effects tested – involving emitted or received aggression, PC1, PC2, body condition and rTL – were not significant (see **Table 2** for statistics). Regarding the correlation between independent variables we found no significant correlation: PC1-PC2 ($r < 0.001$, $t < 0.001$, $p = 1$), PC1-body condition ($r = -0.15$, $t = -0.90$, $p = 0.37$), PC2-body condition ($r = 0.21$, $t = 1.23$, $p = 0.22$), received aggression-emitted aggression ($r = 0.21$, $t = 1.28$, $p = 0.21$). The dependent and independent variable showed no concerning sign of collinearity since the highest VIF (PC1) was of 1.18 (suggested cut-off of 3: Zuur *et al.* 2010).

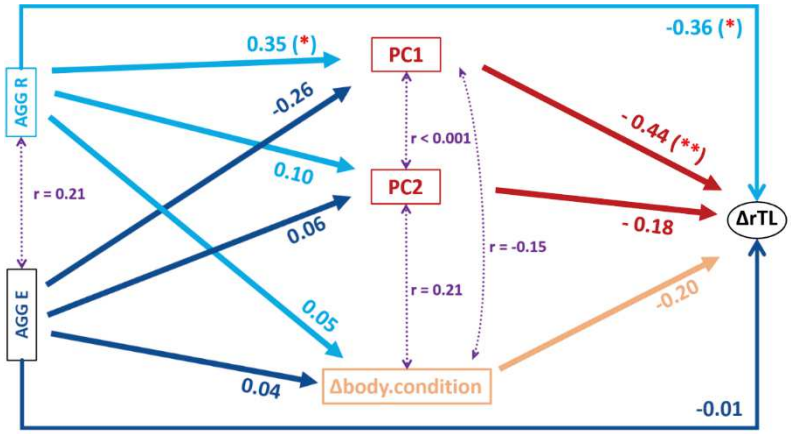


**Figure 3: Path analysis.** Values above the solid arrows are estimates of the models detailed in Table 2. One bright red star in brackets indicates a p-value < 0.05, two indicate p < 0.01 and none indicates a not significant relationship. Values above double-headed dashed arrows are correlation coefficient. None was significant (see main text). *PC1* and *PC2* are aggregated values of oxidative status indicators. These oxidative status indicators measure the variation over the course of the experiment of some biologically meaningful molecules (see main text). *ΔrTL* and *Δ body.condition* are the variation between the start and end of the experiment in, respectively, relative telomere length and body condition. AGG R and AGG E are respectively the number of aggressive behaviours received and emitted, divided by the time a bird was observed. For all models: N = 36 and $VIF_{max}$ = 1.18.

**Table 2: Statistics table of the models used in the path analysis.** (N = 36) A dashed line separates the successive models. Significant values are in bold. *Δ Telomere Length*: variation in telomere length at the experiment term (long). *PC1* and *PC2*: respectively first and second principal component used as global indicator of the changes in bird oxidative status between the start and the end of the experiment. PC1 is positively correlated to the three physiological measurements used and PC2 is positively correlated to OXY but negatively to ROM. *Δ body condition*: variation in body condition at the experiment term (64 days long). Total number of received and emitted aggressions are divided by the time a bird was observed. *CI*: 95% confidence interval. CI for intercepts are not strictly identical but differ only from the fourth digit.

| Dependent variable | Independent variables | Estimates | CI | p-value |
|---|---|---|---|---|
| Δ Telomere Length | (Intercept) | $-2.59E^{-17}$ | [-0.33 ; 0.33] | 1 |
| | PC1 | **-0.44** | **[-0.75 ; -0.13]** | **0.007** |
| | PC2 | -0.18 | [-0.49 ; 0.13] | 0.236 |
| | body_condition_corrected | -0.20 | [-0.51 ; 0.12] | 0.211 |
| PC1 | (Intercept) | $-9.25E^{-17}$ | [-0.33 ; 0.33] | 1 |
| | received aggressions | **0.35** | **[0.01 ; 0.69]** | **0.041** |
| | emitted aggressions | -0.26 | [-0.60 ; 0.07] | 0.121 |
| PC2 | (Intercept) | $1.31E^{-17}$ | [-0.33 ; 0.33] | 1 |
| | received aggressions | 0.10 | [-0.26 ; 0.46] | 0.562 |
| | emitted aggressions | 0.06 | [-0.30 ; 0.42] | 0.737 |
| Δ body condition | (Intercept) | $3.00E^{-17}$ | [-0.33 ; 0.33] | 1 |
| | received aggressions | 0.05 | [-0.31 ; 0.41] | 0.793 |
| | emitted aggressions | 0.04 | [-0.32 ; 0.40] | 0.83 |
| Δ Telomere Length | (Intercept) | $1.45E^{-17}$ | [-0.33 ; 0.33] | 1 |
| | received aggressions | **-0.36** | **[-0.70 ; -0.02]** | **0.038** |
| | emitted aggressions | 0.01 | [-0.33 ; 0.35] | 0.95 |

*3.4. Effects of the social density treatment on bird seed consumption and beak colouration*

On average, bird daily consumption of seed types depended on the social density treatment (significant interaction density x seeds: GLMM: $F = 36.35$, $p < 0.001$). Birds in the high social density treatment consumed 29.9% more antioxidant rich red millet ($p < 0.001$) and 17.2% less yellow panicum ($p < 0.001$) than birds in the low social density condition (**Figure 4**, **Table 3**).

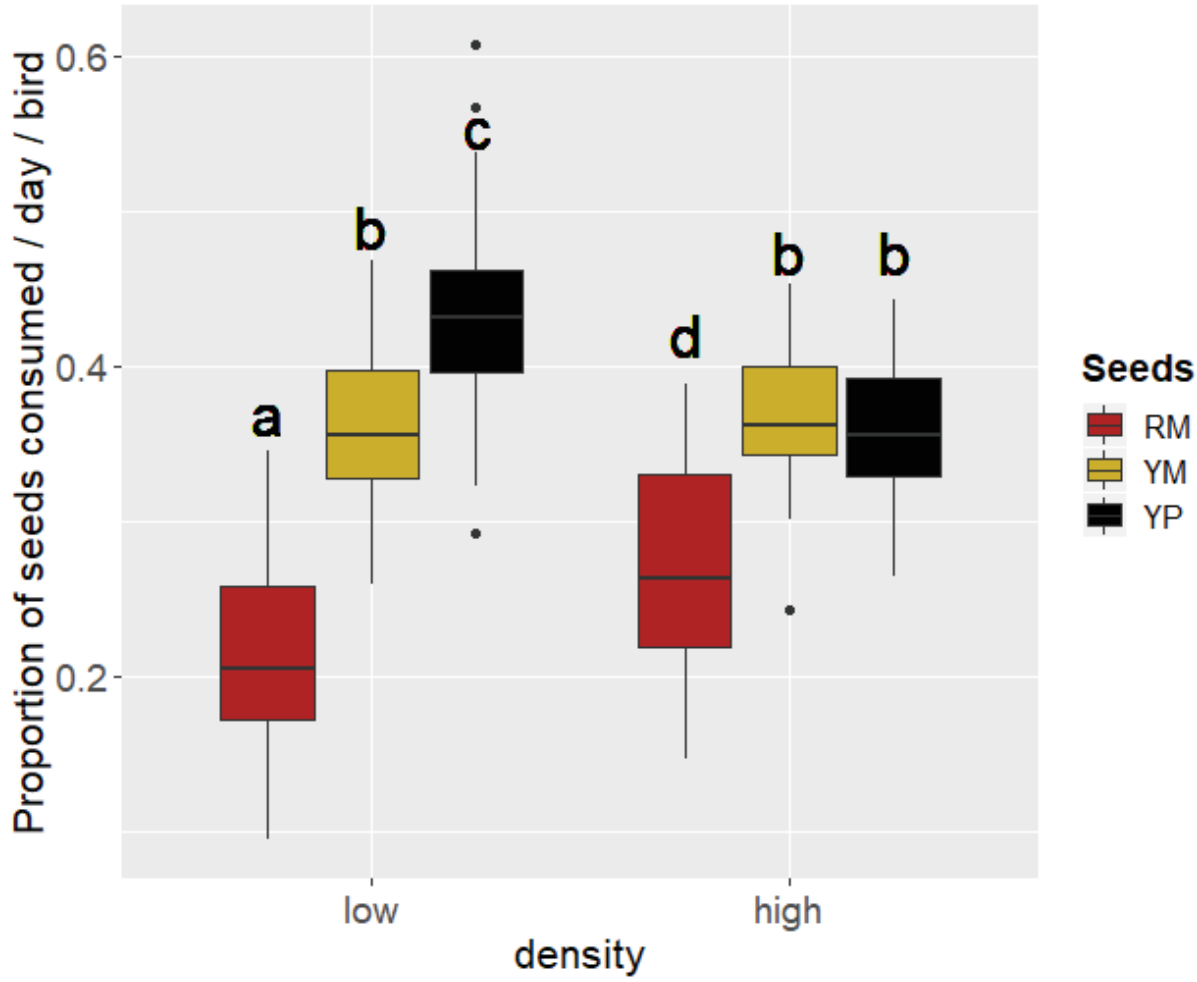


**Figure 4: Effects of social density on seed consumption.** We compared the proportion of seeds consumed per day per bird depending on the social density (high/low) and the type of seeds. Values of the differences between groups, confident intervals and p-values are available in Table S1. Values not sharing the same letter in the figure are significantly different for $P < 0.05$.*RM = red millet, YM = yellow millet and YP = yellow panicum.*

**Table 3: Statistical values for the pairwise comparisons of the interaction between the social density and type of seed.** YM = yellow millet, RM = red millet, YP = yellow panicum. Below, are given the difference between the two modalities of the interaction (diff), the lower (lwr) and upper (upr) limits of the 95% confidence interval and the p-value, adjusted for multiple pairwise comparisons (P-adj from the Tukey's Honest Significant Difference (HSD) test) . Significant differences are in bold and not significant in italic.

| | diff | lwr | upr | P-adj |
|---|---|---|---|---|
| *low:YM-high:YM* | *-0.011* | *-0.044* | *0.023* | *0.95* |
| **high:RM-high:YM** | **-0.094** | **-0.128** | **-0.061** | **<0.001** |
| **low:RM-high:YM** | **-0.157** | **-0.191** | **-0.124** | **<0.001** |
| *high:YP-high:YM* | *-0.012* | *-0.045* | *0.022* | *0.92* |
| **low:YP-high:YM** | **0.062** | **0.029** | **0.096** | **<0.001** |
| **high:RM-low:YM** | **-0.084** | **-0.117** | **-0.050** | **<0.001** |
| **low:RM-low:YM** | **-0.147** | **-0.180** | **-0.113** | **<0.001** |
| *high:YP-low:YM* | *-0.001* | *-0.035* | *0.033* | *1.00* |
| **low:YP-low:YM** | **0.073** | **0.039** | **0.106** | **<0.001** |
| **low:RM-high:RM** | **-0.063** | **-0.097** | **-0.030** | **<0.001** |
| **high:YP-high:RM** | **0.083** | **0.049** | **0.116** | **<0.001** |
| **low:YP-high:RM** | **0.156** | **0.123** | **0.190** | **<0.001** |
| **high:YP-low:RM** | **0.146** | **0.112** | **0.179** | **<0.001** |
| **low:YP-low:RM** | **0.220** | **0.186** | **0.253** | **<0.001** |
| **low:YP-high:YP** | **0.074** | **0.040** | **0.107** | **<0.001** |

## 4. Discussion

Our aim was to evaluate the physiological consequences of aggressive social interactions in zebra finches. We video recorded the behaviour of 18 male and 18 female zebra finches, weighed them and took blood samples at the start and end of the experiment. After 64 days of social interactions, we observed that the received aggression had negative effects on bird relative telomere length both directly, and indirectly, via modifications of their oxidative status. On the other hand, emitted aggression had no significant effect on relative telomere length or oxidative status over the course of the experiment. Similarly, variation in bird body condition over the experiment term did not appear to be influenced by aggression, nor was it associated with variation in relative telomere length.

*4.1. Birds behave more aggressivily at high social densities*

When comparing birds from our high *vs.* low social density treatments, we found overall bird aggression (total number of aggressive behaviours per bird and per hour) was higher in the high social density treatment. In addition, received aggression by individual birds (*in*-strength) was also higher. This confirms the notion that social competition increases with social density and is consistent with previous findings that birds are indeed more aggressive in denser social environments, at least in captivity (Poot *et al.* 2012). At least three (non-mutually exclusive) hypotheses might explain high aggressive behaviour when social crowding occurs.

First, individual costs may arise from social instability or rapid changes in social group or social composition. By increasing social density at the start of the experiment compared to previous housing conditions, we created new social groups, forcing individuals to re-establish social relationships and social hierarchy, creating novel sources of potential conflict (Evans 1970; Caryl 1975). Temporary changes in social group composition are indeed known to affect zebra finch performance, for instance diminish foraging efficiency and co-feeding dynamics (Maldonado-Chaparro *et al.* 2018)

Second, increased aggression at high social densities might be related to reproduction. Although no females laid eggs during our study, we observed occasional attempts of copulations and some males began to build nests. The increased aggressiveness observed may have come from sexual arousal, as previously reported in zebra finches (Evans 1970; Case 1986; Ikebuchi and Okanoya 2006). By increasing social density, we heightened the potential for aggressive behaviour related to competition for mate choice, since a larger number of mates were available to choose from and more fights were required for hierarchy establishment and territory defence (Caryl 1975). This is consistent with the observation that received aggression (*in*-strength) was higher in males than females, suggesting they were either the targets of male competitors or the rejects of unwilling females (mate-choice).

Third, social (space-related) crowding may have resulted in increased competition and thus aggression at feeders (Evans 1970; Caryl 1975) and perching sites (Evans 1970). Moreover, higher social density also reduces the space available per bird to sit on perches, where they are more likely to interact and intrude each other personal limits, 5-15 cm (Ikebuchi and Okanoya 2006). Taken together, these hypotheses explain how aggressiveness might have emerged and been heightened in a dense social environment.

*4.2. Subordinate individuals both suffer from direct and indirect effects of aggression*

In line with social stress hypotheses, we predicted that the toll of social aggression should be strongest both in subordinate (receiving high levels of aggression) and dominant (emitting high levels of aggression) individuals. Over the course of the experiment, such individuals were expected to experience increased oxidative stress, resulting in DNA degradation and telomere shortening. The

antioxidant barrier might also be expected to increase to protect the organism from oxidative stress. Our results are partly in line with these predictions. As expected, received aggression was positively related to PC1, which reflected an increase in all plasmatic oxidative status indicators measured over the course of the experiment: OXY (non-enzymatic antioxidants), ROM (general oxidative damage), and 8-oxo-dG (DNA degradation); and PC1 was positively related to telomere attrition over the 64 days of the experiment. This suggests that birds that were the most targeted by aggressive conspecifics suffered from increased oxidative stress (especially DNA damage since 8-oxo-dG contributed the most to PC1, *i.e.* 44%) resulting in higher telomere attrition. Those results are consistent with previous findings that aggression or unstable social hierarchies are often associated with increasing oxidative stress in vertebrates (Costantini *et al.* 2008a; Rammal *et al.* 2010; Wapstra *et al.* 2011; Beaulieu *et al.* 2014b; Nettle *et al.* 2017) and that telomeres are reduced under stressful conditions (reviewed in Oliveira *et al.* 2016), notably because of oxidative stress (Saretzki and Zglinicki 2002; Houben *et al.* 2008). In addition to this indirect effect mediated via changes in birds' oxidative status, received aggression had a direct negative impact on bird relative telomere length attrition. Interestingly, the path coefficient for this direct effect (-0.36) was more than twice as large as the indirect effect (-0.15 = 0.35 x -0.44), suggesting that alternative, non-mutually exclusive, processes relating received aggression to telomere length are at play. First, this direct effect may reflect latent physiological processes not measured in our study. For instance, received aggression is likely to affect glucocorticoid hormones (Creel *et al.* 1996; Creel 2001; Wapstra *et al.* 2011), known to negatively affect telomere dynamics (Carrero *et al.* 2008; Choi *et al.* 2008; Paul 2011; Angelier *et al.* 2017). Second, such an effect might be explained by inter-individual variation in quality. High quality individuals usually perform well in a suite of phenotypic traits displaying higher foraging performance, higher body condition, higher antibody levels, higher reproductive success and longer telomeres (Le Vaillant *et al.* 2015; Criscuolo *et al.* 2018; Angelier *et al.* 2019). High quality individuals are also often at the top of the hierarchy (Haley *et al.* 1994; Zucker and Murray 2010; Chelliah and Sukumar 2013; Georgiev *et al.* 2015; Francis *et al.* 2018) receiving fewer aggressions, and having better access to food resources (Evans 1970; Caryl 1975), also known to be associated with longer telomeres (Paul 2011; Mizutani *et al.* 2013; Young *et al.* 2017; Spurgin *et al.* 2017). Thus, the negative correlation between received aggression and telomere length attrition may also reflect low quality individuals investing poorly in several traits. Males at the top of the hierarchy are known to express the most aggressive behaviour (Bonoan *et al.* 2013) and have more testosterone (Ardia *et al.* 2010). High testosterone levels also ensure the development of more attractive secondary sexual characteristics (Gil *et al.* 1999 and references therein; McGraw *et al.* 2006; Ardia *et al.* 2010). Testosterone could therefore mediate of this co-variation of several traits, at least in males. Contrary to our expectation, we did not find an effect (direct or indirect) of emitted aggression on oxidative stress and telomere erosion. This is surprising

since fighting to stay on top of the social hierarchy might have been expected to generate oxidative costs in a socially crowded environment. Yet our results suggest that the costs of social aggression may be stronger for individuals receiving, rather than emitting, aggressive behaviour.

*4.3. Aggression and body condition*

Received or emitted aggression had no significant effect on changes in bird body condition over the course of the experiment, nor were changes in body condition associated with changes in telomere length. The link between body condition or growth and telomere dynamics has been established during highly energy-demanding life times: *e.g.* development of chicks (Vedder *et al.* 2017, 2018; Monaghan and Ozanne 2018) or reproduction (Le Vaillant *et al.* 2015; Criscuolo *et al.* 2018; Angelier *et al.* 2019). Although copulation attempts occurred, no female laid eggs. In addition, all individuals were adults and therefore had no growth-related costs. Besides, food was distributed ad libitum, which may offset possible mass loss due to stress. Thus, if deleterious consequences of aggression on bird body condition occurred, our results indicate that these were offset by food availability or favourable energy trade-offs (*i.e.* no investment in growth or reproduction).

*4.4. Conclusion*

Whereas social aggression related to territoriality is common in group-living species, and known to present a potent source of social stress in numerous cases (Creel *et al.* 2013), the mechanistic links relating the social environment to telomere attrition are still unclear. Our data highlight adverse consequences for the most assaulted birds by establishing a causal link between aggression, oxidative stress and telomere length. However, our analysis also highlighted that important alternative mechanisms remain to be tested, as is evident by the relative contribution of direct and indirect effects relating social aggression to telomere attrition (see Nettle and Bateson 2017 for a discussion about causative links between telomere length and behaviour). It would be of particular interest in future studies to test for physiological mechanisms supposed to mediate telomere loss in adverse situation (*e.g.* glucocorticoids, inflammation), in order to clarify the exact signalling pathways involved. To deal with oxidative stress and its potentially harmful consequences, living organisms have evolved either (or both) endogenous antioxidant mechanisms and specific behavioural patterns allowing for the consumption of exogenous antioxidants. Our results needed more statistical power but tended to show that zebra finches buffer oxidative damage by an active selection of antioxidants-rich food. To date such an ability has only sparsely been investigated in non-human animals (Senar *et al.* 2010; Roode *et al.* 2013; Beaulieu and Schaefer 2013, 2014; Beaulieu *et al.* 2014a) and deserves greater attention.

**Author contributions**

Designed the study: JB, VAV, MQ, FC; performed the experiments: MQ, CF, JB, VAV, FC; performed the laboratory work: MQ, CF, FC, SZ; analysed the data: MQ, CF, SS, QS; wrote the paper: MQ; all authors commented on the manuscript.

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**Competing Interest:** The authors declare no conflict of interest.

**Data availability:** Until the article is published, dataset and other electronic supplementary materials are available at https://ncloud2.zaclys.com/index.php/s/nLAdQ8Qrm43aAfi > Chapter 1.

# Chapter 2| Contrasting associations between nestling telomere length and pre and postnatal helpers' presence in a cooperatively breeding bird

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La mère du moineau
Lui réclamant son enfant
Poursuit le chat.

The sparrow's mother
Claiming her child
Stalk the cat.

Kobayashi Issa

**Abstract**

In cooperatively breeding birds, non-breeding 'helpers' are supposed to improve the development and survival of offspring, but it can also lead to decreased pre- or post-natal parental reproductive effort. To examine whether prenatal and postnatal helpers influence offspring condition, we conducted an egg cross-fostering experiment in sociable weavers (*Philetairus socius*), chick social environment (presence/absence of helpers) being similar or different from the prenatal environment. We tested whether relative telomere length (rTL), an indicator of somatic maintenance, was influenced by prenatal and/or postnatal presence of helpers 9 and 17 days after hatching, and if rTL predicted long-term fledging survival. Nine days after hatching, we found an overall positive effect of postnatal helpers on rTL: in nests with prenatal helpers, a reduction in the number of helpers post-hatch was associated with shorter telomeres, while in nest without prenatal helpers, an increase in postnatal helpers was associated with longer telomeres. However, when prenatal helpers were already present, an increased number of helpers after hatching led to shorter telomeres. We found that 9-day old chicks with longer rTL were more likely to survive over the 5 years following hatching. Close to fledging (17 days after hatching), there was no detectable effect of the experiment on rTL and no link between rTL and survival. Overall, this study presents experimental partial support for the importance of the presence of helpers for offspring rTL in a wild population of cooperatively breeding birds and on the link between early-life telomeres length and long-term survival in a wild population.

**Keywords:** sociable weaver, cross-fostering, offspring, early social stress, helping-at-the-nest, survival, maternal effect, growth maintenance trade-off

**Introduction**

In iteroparous species, individuals are expected to maximize their lifetime reproductive success through trade-offs between the investments in current reproduction vs. survival and future reproduction (Stearns 1977). For egg-laying species, females may vary the allocation of nutrients, anti-bodies, or other substances to eggs, thereby influencing the environment in which their offspring will develop. This allocation is in turn influenced by the mother's breeding environment, which is expected to be an indication of the environment in which the offspring will develop. In agreement, studies have shown that when females can anticipate the conditions experienced during reproduction they can influence traits such as egg size (e.g. Fox *et al.* 1997a; Taborsky 2006; Vijendravarma *et al.* 2010), and hormonal investment (Groothuis *et al.* 2019), and these traits can correlate with offspring survival or behaviour (e.g. Krist 2011; Paquet *et al.* 2015a).

Among the environmental effects experienced by females and their developing offspring, sociality has been receiving increasing attention (e.g. Russell *et al.* 2007, 2008; Dixit *et al.* 2017). In cooperatively breeding species, sexually mature non-breeding individuals – called 'helpers' – assist in raising the offspring of others by bringing additional food to the nest and performing other tasks such as territory defence (Cockburn 1998). The presence of these helpers is therefore thought to improve the breeding conditions experienced by females and/or offspring, and studies on several species have generally shown that helper presence is associated with improved juvenile condition and survival (e.g. Ridley 2007; Kingma *et al.* 2010; Brouwer *et al.* 2012; Preston *et al.* 2016; Tanaka *et al.* 2018). Breeding females may respond to this improvement in reproductive conditions by either i) increasing their investment in the eggs or embryos to improve the chances of successful reproduction, ii) maintaining their investment, or iii) decreasing it, thereby reducing their cost of reproduction and increasing their survival and future reproduction. Reduction in maternal investment should then be compensated by the additional food brought by helpers (Russell and Lummaa 2009). The direction and magnitude of the response are expected to be influenced by the species' life-history strategy and especially by the effects of maternal investment on mother's survival prospects and chances of breeding successfully in the future (Russell and Lummaa 2009). In birds, cooperatively breeding species tend to be long-lived (Arnold and Owens 1998; Downing *et al.* 2015), and for long-lived species, maximization of lifetime reproductive success was suggested to be more efficiently achieved through increasing the number of breeding events in life than through increased investment in each breeding event (Ghalambor and Martin 2001; Drent and Daan 2002; Reid *et al.* 2003). In agreement, a recent meta-analysis on 10 cooperatively breeding birds highlighted a tendency for mothers to reduce egg size in larger groups in most species studied (Dixit *et al.* 2017).

It is less clear whether such differential maternal investments translate into differences in the condition and survival of offspring and what is the relative influence of the pre- and post-natal environment. For example, in birds, there are indications that larger eggs are positively associated with nestling's body size, growth and survival (Krist 2011). However, these associations could arise from genetic effects (Morrison *et al.* 2009; Voillemot *et al.* 2012), pre- or post-natal maternal effects (Velando *et al.* 2005; Weber *et al.* 2018) and/or from intertwined pre- and post-natal environmental influences (Bize *et al.* 2002). From the offspring's perspective, the presence of helpers can be associated with weaker prenatal maternal egg investment (Paquet *et al.* 2013, 2015a) but identical or higher total feeding rate, as more carers are present. Higher levels of care can lead to increased growth rate and potentially better condition or higher body mass at fledging, and thus increased likelihood to survive the first few months after fledging (Russell *et al.* 2007; Canestrari *et al.* 2011; Loock *et al.* 2017). The prenatal and postnatal effects of helpers' presence can thus exert opposite influences on nestling fitness. Studies of maternal effects concerning the social environment therefore need to investigate the influence of these two distinct environments. This requires i) identifying meaningful proxies of offspring fitness and ii) conducting experiments to separate the influence of the pre- and post-natal environments.

Meaningful proxies of offspring condition should strongly correlate with offspring survival. Telomeres are repeated sequences at the ends of eukaryotic chromosomes. Replication leads to the shortening of the telomeric DNA sequence at each cell division, and beyond a certain number of cell division, telomeres become too short, triggering cell replicative senescence, which participates to the senescence of the whole organism (Tchkonia *et al.* 2013). Consequently, telomeres became increasingly used as predictors of age-related survival. Telomere length (TL) and/or telomere shortening rate have been shown to predict adult survival in numerous studies involving bird species (Pauliny *et al.* 2006; Bize *et al.* 2009; Salomons *et al.* 2009; Heidinger *et al.* 2012; Barrett *et al.* 2013; Boonekamp *et al.* 2014). Both ecological (Geiger *et al.* 2012; Stier *et al.* 2014; Salmón *et al.* 2016; Spurgin *et al.* 2017) and social (Hall *et al.* 2004; Nettle *et al.* 2015; Hammers *et al.* 2019) environments may increase or decrease TL (see Dugdale and Richardson 2018 for a review of the environment influence on vertebrate telomere length). Although not all studies have established a clear link between telomere length and longevity parameters (e.g. Beaulieu *et al.* 2011; Caprioli *et al.* 2013; Reichert *et al.* 2014b), the general trend in birds, mammals and reptiles is towards a consistent positive association between long telomeres and survival (Wilbourn *et al.* 2018). Taken together these studies support the use of telomeres as reliable indicators of longevity and/or individual quality, sensitive to early ecological and social stress, especially when measured during early life in birds (Eastwood *et al.* 2019).

In this study, we conducted a cross-fostering experiment to investigate how the pre- and post-natal social environments influence offspring condition, measured by relative telomere length (rTL). In addition, we controlled for a potential trade-off suggested in the literature (e.g. reviewed in Monaghan and Ozanne 2018)

between telomere length (related to self-maintenance) and mass or body condition (related to growth). We further assessed the relationship between rTL and survival by testing whether rTL predicts juvenile apparent survival as estimated with capture-mark-recapture models.

Our study model was the sociable weaver (*Philetairus socius*), a colonial cooperatively breeding passerine endemic to the semi-arid Kalahari savannahs of southern Africa. These weavers are long-lived among passerines (up to 16 years, Covas 2012) and nest predation by snakes leads to an extremely low reproductive success (Covas *et al.* 2008). A previous study found that in sociable weavers egg mass and egg content was negatively associated with the number of helpers at the nest (Paquet *et al.* 2013). In our cross-fostering experiment, we transferred whole clutches to a foster nest (see cross-fostering design in Table 1). The nestlings might thus have been conceived in the presence of helpers but raised in their absence (or vice-versa) and thus their prenatal social environment may differ from the post-natal social environment. Based on the previous knowledge of this species' life-history, and a previous result showing that females with helpers lay smaller eggs with lower levels of steroid hormones than females without helpers (Paquet *et al.* 2013), we predict : (i) a negative effect of the prenatal presence of helpers on chick rTL; (ii) a positive effect of the postnatal presence of helpers on chick rTL. In addition, we expect an interaction between pre- and post-natal helper presence, and we predict (iii) longer rTL of chicks that benefit from both higher maternal investment in eggs (absence of pre-hatching helpers) and additional care provided by an increased postnatal helper presence; (iv) a stronger negative impact on chick rTL when the postnatal social environment is unfavourable (decreased presence of postnatal helpers), while prenatal conditions were unfavourable too (presence of prenatal helpers). Finally, we expect that telomere length will be positively associated with survival after fledging.

**Material and Methods**

*Species and study area*

The study was conducted at Benfontein Game Farm in the Northern Cape Province, South Africa (approx. 28°53'S, 24°89'E). The study area covers 15 km² and contains approximately 30 sociable weaver colonies. The vegetation is Kalahari sandveld, consisting of open savannah dominated by *Stipagrostis* grasses and the camelthorn tree *Acacia erioloba*. The area is semi-arid, experiencing low and unpredictable rainfall (average 431± 127 mm/year, C.V. = 35.4; Weather Bureau, Pretoria). Most of the precipitation in the study area falls during the summer months, from September to April, when breeding usually takes place.

Sociable weavers are small (ca. 27g) sexually monomorphic passerine birds endemic to southern Namib and Kalahari regions of Southern Africa. They build a large communal nest made of numerous independent chambers, wherein the birds roost throughout the year and breeding takes place (Maclean 1973a). These

weavers are cooperative breeders, with up to six individuals assisting a breeding pair in raising the chicks. Helpers are most often the offspring of one or both breeders (Covas *et al.* 2006). Younger helpers are both males and females while, when older than one year, helpers are usually males (Doutrelant *et al.* 2004). There is no extra-pair paternity in this species (Covas *et al.* 2006; Paquet *et al.* 2015b). Sociable weavers feed mostly on insects, but also seeds and other plant products (Maclean 1973b).

*Data collection*

Data were collected between September 2013 and February 2014 (for the variables related to breeding, growth, and telomeres) and until 2019 for chick survival. Unique numbered aluminium ring and colour combination are attributed to each adult during the annual captures in winter and to each nestling before fledging, allowing individual identification of each bird. During the breeding season, all chambers in the study colonies are checked every three days, allowing us to identify new clutches and estimate clutch size. All chambers are visited daily at the end of the incubation period (approximately 15 days), in order to identify the hatching date. Two additional sets of measurements were performed nine days (D9) and seventeen days (D17) after the first chick hatched in each nest. Hence, chicks were captured only twice, limiting stress. D9 is the earliest moment where all chicks can be ringed and sampled for blood and D17 was chosen to have a measure as late as possible before fledging. At these two dates, we measured brood size (number of chicks) and nestling mass, and we took blood samples from the brachial vein for sex determination and telomere analysis. This procedure was conducted on a total of 132 nestlings distributed among 15 colonies (containing approximately 400 chambers).

Blood samples at D9 were stored in 95% ethanol. In contrast, because of use in other experiments, blood samples at D17 were centrifuged immediately and red blood cells were stored frozen. Because different storage conditions may affect rTL measured via quantitative polymerase chain reaction (qPCR, Reichert *et al.* 2017), we restricted our analysis to same nestling age comparisons, storage conditions within samples from the same nestling age being strictly identical and could not affect the outcomes. Tarsus length at D17 was also recorded and used to build the body condition. The body condition of one chick was calculated as the residual value coming from the regression between tarsus length and mass at D17 (regression plot and statistics available in Electronic Supplementary Material, **ESM 1**). Tarsus length was not measured at D9. Observations took place from under a hide placed at 3-5 m from the colony. The 62 nests from which the 132 chicks in this study originated are monitored annually, allowing us to analyse the relationship between the early rTL and survival from birth to the present (6 years).

*Cross-fostering procedure*

The detailed methods can be found in Paquet *et al.* (2015a), with the difference that here we focused on the reproduction period from September 2013 to February 2014. In brief, when two clutches from the same colony and of the same size were laid synchronously (or within a 1-day interval), both whole clutches were swapped on the day after the last egg was laid. Group sizes after hatching (*i.e.* parents + number of postnatal helpers) were established by counting the number of birds seen feeding the chicks. The group size before hatching can be predicted form the group size observed after hatching (Paquet *et al.* 2016), allowing us to infer the number of helpers before cross-fostering retrospectively. Within the 132 nestlings sampled during this study, 72 were involved in the cross-fostering protocol, and among them, the presence of helpers was known for 50 of them (18 broods). The pre- and post-natal social environments for these 50 chicks are detailed in **Table 1**.

**Table 1: Cross-fostering procedure design.** Immediately after hatching, the whole clutch was transferred from its original nest with or without helpers to a foster nest with or without helpers, resulting in chicks with similar or different pre- and post-hatching social environment. Chicks were swapped within the same colonies. 50 chicks were involved in this procedure, the sample sizes for nestlings and nests where they come from can be read within the brackets in bold font. In bold are also indicated the corresponding experimental groups (EG; +1: increased number of helpers, 0: no change, -1: decreased number of helpers).

| | | **Foster nest (postnatal)** | |
|---|---|---|---|
| | | **without helpers** | **with helpers** |
| **Original nest (prenatal)** | **without helper** | *never any helper*<br>**(9 chicks, 4 nests)**<br>**EG: 0 (n=9)** | *postnatal helper(s) only*<br>**(11 chicks, 5 nests)**<br>**EG: +1 (n=11)** |
| | **with helpers** | *prenatal helper(s) only*<br>**(12 chicks, 5 nests)**<br>**EG: -1 (n=12)** | *always at least one helper*<br>**(18 chicks, 9 nests)**<br>**EG: +1 (n=8) / 0 (n=4) / -1 (n=6)** |

*Telomere length measurements*

To assess the prenatal and postnatal effect of helpers on chick rTL, we measured early relative telomere length at D9 and just before fledging at D17 in chicks using the qPCR procedure. This protocol was first developed by Cawthon (Cawthon 2002) and thereafter popularised in birds' studies (Criscuolo *et al.* 2009; Herborn *et al.* 2014; Nettle *et al.* 2015; Meillère *et al.* 2015; Costanzo *et al.* 2017). The qPCR provides a non-absolute measure of an individual's TL, a relative telomere length as it is expressed relatively to one specific sample that is given

arbitrarily the value of 1 (see Nussey *et al.* 2014; Lai *et al.* 2018) for a comparison between TL measurement methods). Shortly, after genomic DNA extraction from red blood cells (nucleated in birds) using silica-membrane columns (Nucleospin Blood QuickPure, Macherey-Nagel, Düren, Germany), DNA quantity and quality were assessed based on spectrophotometer absorbance (Nanodrop 1000 Thermo Scientific, ratios A260/280 and A260/230) and gel-migration (on a sub-sample randomly chosen of 30 samples, see **ESM 2**). Dilutions of extracted DNA (at 2.5 ng/uL) were prepared using sterile distilled water and were amplified using telomere and control gene primers with the BRYT Green fluorescent dye (GoTaq qPCR Master Mix, Promega Charbonnières-les-Bains, France). We used recombination activating gene RAG1 as our control gene for weavers, designed to be non-variable among the individuals of our population (as advised by Smith *et al.* 2011; see ESM 2). The forward and reverse primers for the control gene were: SOWEA-F: 5'-TGCAAAGAGATTTCGATATGATG-3', SOWEA-R: 5'-TCACTGACATCTCCCATTCC-3'. Tel1b: 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3', Tel2b: 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3' for the telomere amplification. An automated thermocycler (CFX 384 Biorad Hercules, USA) was used, with reaction conditions set at 95°C for 2 min, followed by 40 cycles of data collection at 95°C for 1 min and a 60°C annealing–extension step for 1 min for RAG1. For the telomeric sequence, the conditions were set at 95°C for 2 min followed by 30 cycles of data collection for 15 s at 95°C, 30 s at 56°C, and 1 min at 72°C. The 132 weaver samples used in the present study were part of 504 samples that were measured in duplicates on 5 different runs. Each run includes 1 plate of telomere sequence amplification and 1 plate of control gene sequence amplification, due to the differences in qPCR temperature conditions. On each plate, we ran a dilution curve (20 to 0.313 ng) to check that the efficiency of amplification did not vary among plates, and a melting curve to check for amplification artifacts (like primer-dimer signal, see **ESM 2**). The mean qPCR amplifying efficiencies were 99.8 % (99.4-99.9) for telomere and 99.9 % (99.7-100.1) for the control gene (100 % reflect a doubling of the DNA sequence at each amplification step). Samples were randomly distributed over the plates, and 40 were repeated to assess inter-plate variation. As advised by Eisenberg (2016), rather than coefficients of variation, we reported the interplate and intraplate intra-class correlation coefficients for T/S ratio, respectively 0.895 and 0.767.

The rTL is then calculated from the T/S ratio, where 'T' is the copy number of the telomeric sequence and 'S' is the copy number of the control sequence. We took into account the slight variation of efficiency between telomere and RAG1 amplifications by calculating the T/S ratio accordingly to Pfaffl's recommendation (Pfaffl 2001):

$[(1+E_{(telomere)})\ ^{\wedge}\ \Delta Cq_{(telomere)}] / [(1+E_{(RAG1)})\ ^{\wedge}\ \Delta Cq_{(RAG1)})]$ where'E' is the amplification efficiency and '$\Delta$Cq' the difference in time required to reach the fluorescence detectability threshold between control and sample (Cqcontrol - Cqsample).

*Statistical analysis*

Categorical variables were tested through ANOVA (package 'stats' v.3.6, R Core Team 2019). Post-hoc comparisons were done using the *multcomp* R package with Tukey contrasts corrections (Hothorn *et al.* 2008). Quantitative variables including the T/S ratio were first z-scored and then tested directly through the t statistics provided by default by the function 'lmer'. Statistical tests were performed by the R software (R Core Team 2019) at the significance threshold $\alpha$ = 5%.

*Effects of helpers on chick telomere length: definition of variables*

In our analysis, we considered the effects of (i) the natural presence of helper in the nest of origin (hereafter referred to as 'prenatal presence') and (ii) the postnatal change in helper presence induced by the cross-fostering (hereafter referred to as 'postnatal helper change'). The prenatal presence of helpers was coded 0 (no helpers) and 1 (presence of helpers) while the postnatal helper change was coded 0 (no change), -1 (reduced number of helpers) and +1 (presence or increased number of helpers).

We tested if the prenatal presence of helpers and the postnatal helper change, as well as their interaction had an impact on early telomere length (i) nine days after hatching and (ii) later, just before fledging, at seventeen days after hatching. Telomere length and dynamics are known to be affected by individual characteristics, such as sex (Barrett and Richardson 2011; Young *et al.* 2013) or growth rate (Ringsby *et al.* 2015; Monaghan and Ozanne 2018; Vedder *et al.* 2018), as well as by the social environment (Hall *et al.* 2004; Nettle *et al.* 2015; Dugdale and Richardson 2018; Hammers *et al.* 2019). Hence, in addition to the prenatal presence and the postnatal helper change, we tested the influence of the following explanatory variables: sex, breeding period (different climatic conditions), brood size (intra-nest social competition), and body condition (an indicator of chick growth). The breeding period had two levels: the first between October and November 2013 (sparse rains) and the second between December 2013 and January 2014 (abundant rains). The prenatal presence of helpers and the postnatal helper change were not correlated: ($\chi^2$ = 0.040, p = 0.842), which validates the randomness of the cross-fostering procedure. Moreover, there was no evidence for collinearity between all other variables (Variance Inflation Factors were less than three: $VIF_{max}$ = 1.49).

*Effects of helpers on chick telomere length: model fitting and selection*

For the tests conducted either at day 9 or day 17, we built separated mixed linear models (LMM) with 'nest' as a random factor nested in 'colony' to control for non-independence of chicks in a nest. Due to the relatively low sample size (n=50), we decided not to include more than five explanatory variables to avoid over fitting (Harrison *et al.* 2018). We applied a model selection procedure with the 'MuMIn package on R, based on the lowest Aikake's information criterion corrected for small sample sizes (AIC; v. 1.43 Bartoń 2013). The procedure

was adjusted to systematically provide models containing the prenatal presence and postnatal helper change and a maximum of five variables including the two variables concerning the helper presence. We chose models as complete as possible with a maximum ΔAIC of 2. Then, depending on the selection procedure, the interaction term 'prenatal presence x postnatal helper change' may not be included in the lowest AIC model. All proposed and selected models involving telomere length are available in electronic supplementary material (**ESM 3**, **Table S1**). We graphically ensured that model residuals did not significantly differed from a Gaussian distribution and related Anderson-Darling's tests had *p-value* > 0.05 for rTL both 9 and 17 days after hatching, respectively A = 0.25, p = 0.72 and A = 0.33, p = 0.51. A Pearsons' correlation test was conducted to test for correlation of intra-individual values of chick telomere lengths repeatedly measured at day 9 and day 17 post-hatch.

*Chick body condition and body mass growth vs. telomere length*

Chick body condition was calculated as the residuals of the regression of body mass vs. tarsus length both measured at D17. Using the same statistical procedure as for telomere length, we tested whether the effect of helpers could be reflected in the body condition of chicks through mixed linear models with mass at D9 and body condition at D17 as dependent variables. Full models used in the model selection procedure included brood sizes at D9 or D17, chick sex and season, as well as nest / colony as random factors. Furthermore, we used the same statistical approach to examine the link between body condition at D17, body mass at D17 or body mass gain between D9 and D17 and chick telomere length at D17.

*Telomere length and apparent survival*

To estimate whether rTL predicted apparent survival we fitted Cormack-Joly-Seber (CJS) models (Gimenez *et al.* 2007) on six years of capture-mark-recapture history of 132 fledglings (59 females and 73 males from cross-fostered as well as non-cross-fostered nests). The CJS model is composed of apparent survival probability $\phi$ (defined as the probability to survive and not permanently emigrate the next year) and recapture probability $\mathbf{p}$ (defined as the probability of an individual apparently alive in a year to be recaptured with mist nets that year). We set $\phi$ to vary linearly with rTL (scaled) on the logit scale. We also allowed both $\phi$ and $\mathbf{p}$ to vary with the sex of the individuals as we expected them to be lower for females (Dijk *et al.* 2015; Paquet *et al.* 2015b) and to make sure that any relationship between rTL and apparent survival was not solely due to sex differences.

We specified both capture-recapture models using JAGS, version 4.2.0 (Plummer 2015) run using the rjags package (Plummer 2013) in Program R, version 3.4.3 (see **ESM 4** for JAGS code). We estimated parameters using vague priors (see **ESM 4** for priors and initial values). We used 3000 posterior samples from three Markov Chain Monte Carlo (MCMC) chains based on 3000 iterations after an adaptation period of 5000, a burn-in of 10000 and thinning interval of 3. We assessed model convergence both visually and by using the "R hat" Gelman–Rubin

statistic (Gelman and Rubin 1992) and found the 95% upper limits of potential scale reduction factors to equal 1 for all estimated parameters, indicating that convergence was achieved. We assessed the fit of our two models to the data (*i.e.* posterior predictive checks) by simulating capture recaptures histories from our two models at each iteration and comparing the number of simulated recaptures for the 3000 posterior samples with the number of observed recaptures. Simulated values systematically lower or higher than observed values would indicate a lack of fit (*i.e.* p(simulated < observed) close to 0 or 1). We obtained p(simulated < observed) = 0.47 and 0.49 for models looking at rTL at D9 and D17 respectively, indicating a good fit to the data.

*Ethical statement*

All experiments were conducted with permission from the Northern Cape Department of Tourism Environment and Conservation (permit FAUNA 942/2012) and the approval of the Ethics Committee of the University of Cape Town (2009/V12/RCREN). Our procedures involved the capture, confinement, handling, and blood sampling of the birds in the field, with the time elapsed between extracting the birds from the nets until the last bird was released ranging from 2 to 3 h. While queuing to be processed, birds rested in individual bird bags and were placed in a quiet, ventilated, and shaded area. The sampling volume (ca. 75 μl) remained well below the prescribed limits for the percentage of the total blood volume of this passerine. After handling, the adult birds could recover for a few minutes before being released in small groups. Any birds that showed signs of fatigue or injury were taken to an indoor aviary to recover and were subsequently released. This happened for less than 1% of the birds handled. To decrease handling times, captures were conducted with a team of 8-12 experienced ringers that were allocated specific tasks to streamline the procedures conducted.

**Results**

We present for each result below the estimate ± standard error in brackets. In Table 2 (telomere-related models) and Table 3 (body mass-related models), we present in addition 95% confidence interval (CI95%), test statistics and *p-values*. After the model selection procedure (detailed in **ESM 3**, **Table S1**), all models contained the following independent variables: prenatal presence, postnatal helper change and the random effect of the nest nested in the colony. The interaction between pre- and post-natal helpers (prenatal presence x postnatal helper change), as well as chick sex and brood size were kept only for the presence of helpers at D9. Only the breeding season was kept as an additional fixed factor for the model testing individual variability in rTL at D17. The original data sets regarding both the effect of helpers on chick rTL and the effect of rTL on survival are available online as distinct tables in **ESM5.** Chicks rTL measured at D9 and D17 were found to be significantly correlated – even if the correlation is not high ($r = 0.27$, $t = 2.64$, $p = 0.009$, $n = 93$, Pearson's correlation).

*Effect of the prenatal and postnatal presence of helpers on chick rTL*

The detailed statistics are given in **Table 2** and related plots are **Figure 1A for D9 and 1B for D17**. Nine days after hatching, we found a significant interaction between the prenatal helper presence and the postnatal helper presence (-1.76 ± 0.54). The Tukey contrasts post-hoc comparisons showed that for the chicks from nest without helpers during the prenatal stage, obtaining postnatal helpers was associated with an increase in their rTL nine days after hatching (0/1 vs 0/0, post-hoc: 1.54 ± 0.49, z = 3.13, p = 0.01). In addition, a decrease in the number of helpers was associated with a decrease in the rTL for chicks that had prenatal helpers (1/-1 vs 1/0, post-hoc: -1.21 ± 0.44, z = -2.75, p = 0.042). However, the rTL of chicks in nest with prenatal helpers was not affected by an increase in helper number after hatching (1/+1 vs 1/0 and 1/-1, post-hoc: respectively -1.23 ± 1.20, z = -1.03, p = 0.83 and 1.20 ± 0.51, z = 2.35, p = 0.12). Among the chicks experiencing additional helpers, those with prenatal helpers had shorter telomeres on average (0/+1 vs 1/+1, post-hoc: -1.18 ± 0.44, z = -2.73, $p$ = 0.043). The effect of chick sex and brood size were not significant (respectively -0.43 ± 0.25 and 0.09 ± 0.17). When measured at D17 post-hatch, neither the prenatal presence or the postnatal helper change nor their interaction term were found to be significantly related to offspring's rTL (**Figure 1B, Table 2B**). The breeding season, while kept in the selected model, had no significant effect (0.12 ± 0.18).

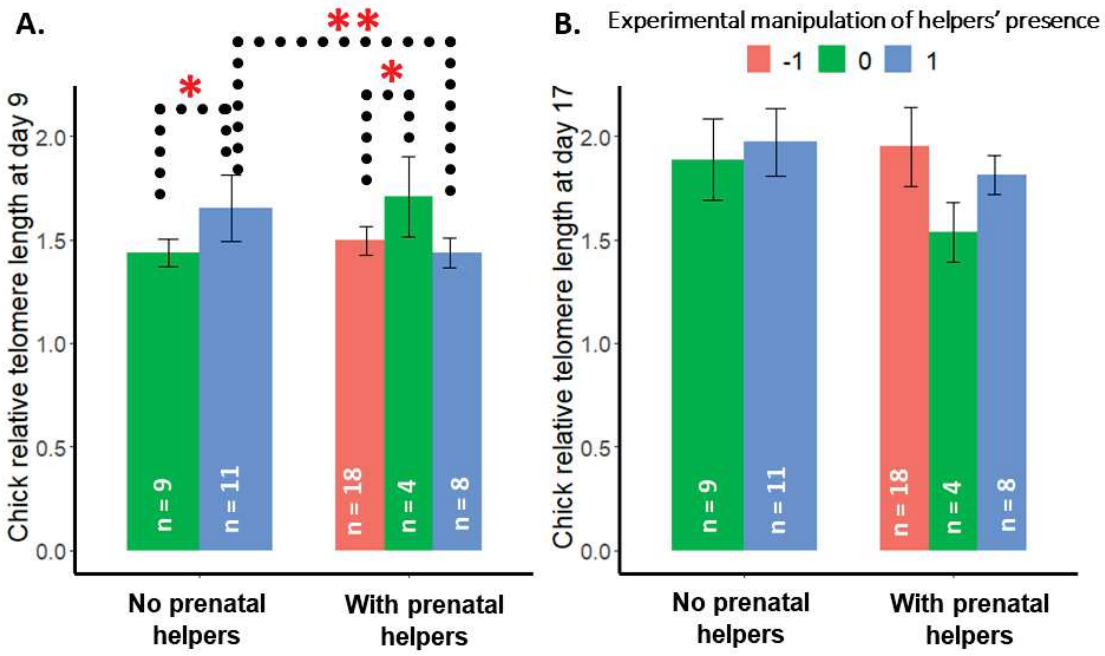


**Figure 1: Relative telomere length (rTL, T/S ratio) 9 days (A) and 17 days (B) after hatching in relation to helpers' presence.** We compared the rTL of 50 cross-fostered individuals according to (i) the presence of helpers before the cross-fostering and (ii) after the cross-fostering. This latter experimental factor is categorised as: increased number of helpers (+1, blue); no change in helpers' presence (0, green) and decreased number of helpers (-1, red). **(A)** Post-hoc significant differences (Tukey contrasts) concerned: longer rTL in chicks that started with no helpers but get experimental helpers presence ($p$ = 0.010); decreased rTL in chicks that started with helper presence but thereafter experienced no helpers ($p$ = 0.042); shorter rTL in chicks that started with helpers and experienced increased helpers presence after hatching compared to chicks that had helpers only after hatching ($p$ = 0.043). **(B)** no significant differences were found at D17. See text for statistics. White numbers inside the bars give the sample size.

**Table 2: Effect of the prenatal and postnatal presence of helpers on chick relative telomere length** (rTL, z-transformed). The following tables list the estimates ('est.'), 95% confidence interval ('CI95%'), standard error ('SE'), test statistics (t or F respectively for continuous or categorical variables) and p-values ('*p*') from linear mixed models. We only consider the variables retained after the model selection procedure (ESM 3). Continuous variables have been scaled and transformed into z-scores. Lines in bold indicate a significant test with α = 0.05 as a threshold. Results are given for chick telomere length at 9 **(A)** and 17 **(B)** days after hatching. In this latter case, random effects did not explain a significant part of the variance.

**A)** **rTL D9**

| *Predictors* | *Estimates* | *std. Error* | *CI* | *Statistics* | *p* |
|---|---|---|---|---|---|
| Intercept | -0.08 | 0.55 | -1.17 – 1.00 | -0.15 | 0.88 |
| Prenatal presence of helpers | 0.29 | 0.45 | -0.58 – 1.17 | 0.65 | 0.513 |
| Postnatal helper change | 1.18 | 0.5 | 0.20 – 2.16 | 2.36 | **0.018** |
| Brood size D9 | 0.09 | 0.17 | -0.24 – 0.43 | 0.54 | 0.587 |
| Chick sex | -0.43 | 0.25 | -0.91 – 0.05 | -1.74 | 0.082 |
| Prenatal presence *x* Postnatal helper change | -1.76 | 0.54 | -2.81 – -0.70 | -3.27 | **0.001** |
| **Random Effects** | | | | | |
| **$\sigma^2$** | 0.46 | **$\tau_{00}$ (nest:colony_id)** | 0.5 | **Observations** | 50 |
| **N (nest)** | 18 | **$\tau_{00}$ (colony_id)** | 0.39 | **Marginal $R^2$ / Conditional $R^2$** | 0.177 / 0.718 |
| **N (colony_id)** | 3 | **ICC** | 0.66 | | |

**B)** **rTL D17**

| *Predictors* | *Estimates* | *std. Error* | *CI* | *Statistics* | *p* |
|---|---|---|---|---|---|
| Intercept | 1.91 | 0.17 | 1.57 – 2.25 | 11.04 | **<0.001** |
| Prenatal presence of helpers | -0.13 | 0.21 | -0.53 – 0.28 | -0.61 | 0.542 |
| Postnatal helper change | -0.06 | 0.12 | -0.29 – 0.18 | -0.48 | 0.635 |
| Breeding season | 0.12 | 0.18 | -0.23 – 0.46 | 0.66 | 0.508 |
| **Random Effects** | | | | | |
| **$\sigma^2$** | 0.39 | **τ00 (nest:colony_id)** | 0 | **Observations** | 50 |
| **N nest** | 18 | **τ00 (colony_id)** | 0 | **Marginal $R^2$ / Conditional $R^2$** | 0.017 / NA |
| **N (colony_id)** | 3 | | | | |

*Effects of helpers on chick body mass and body condition*

Neither the prenatal or the postnatal presence of helpers were associated with a change in chick body mass at D9, or chick body condition at D17 (Table 3). Only brood sizes (D9 and D17) negatively affected body mass at D9 and body condition at D17, respectively. Testing body mass at D17 or the gain in body mass between D9 and D17 did not shown any significant relationships (data not shown). There was no relationship between body mass gain (D17 – D9) and chick rTL at D17 (ESM 6, Table S2).

Table 3: Effect of the prenatal and postnatal presence of helpers on the chick mass at day 9 (A) and chick body condition at day 17 (B) post-hatch (linear mixed models). None shows a significant effect of helpers. Testing body mass at D17 or body mass gain between D9 and D17 led to similar non-significant effects (data not shown).

**A) Body mass D9 (g)**

| *Predictors* | *Estimates* | *std. Error* | *CI* | *Statistic* | *p* |
|---|---|---|---|---|---|
| Intercept | 2.01 | 0.99 | 0.06 – 3.96 | 2.02 | **0.044** |
| Prenatal presence of helpers | 0.51 | 0.43 | -0.32 – 1.35 | 1.2 | 0.231 |
| Postnatal helper change | 0.14 | 0.59 | -1.00 – 1.29 | 0.24 | 0.808 |
| Brood size D9 | -0.88 | 0.23 | -1.34 – -0.42 | -3.77 | **<0.001** |
| Chick sex | 0.51 | 0.26 | -0.01 – 1.02 | 1.94 | 0.053 |
| Prenatal presence x Postnatal helper change | 0.35 | 1.09 | -1.79 – 2.49 | 0.32 | 0.752 |
| **Random Effects** | | | | | |
| **$\sigma^2$** | 0.62 | **τ00 (nest:colony_id)** | 0.11 | **Observations** | 50 |
| **N (nest)** | 18 | **τ00 (colony_id)** | 0.46 | **Marginal $R^2$ / Conditional $R^2$** | 0.255 / 0.611 |
| **N (colony_id)** | 3 | **ICC** | 0.48 | | |

**B) Body condition D17**

| *Predictors* | *Estimates* | *std. Error* | *CI* | *Statistic* | *p* |
|---|---|---|---|---|---|
| Intercept | -0.99 | 0.5 | -1.97 – 0.00 | -1.95 | 0.051 |
| Prenatal presence of helpers | 0.36 | 0.4 | -0.42 – 1.15 | 0.9 | 0.367 |
| Postnatal helper change | 0.9 | 0.44 | 0.04 – 1.77 | 2.04 | **0.041** |
| Breeding season | 0.55 | 0.28 | -0.00 – 1.11 | 1.95 | 0.051 |
| Chick sex | 0.16 | 0.21 | -0.26 – 0.58 | 0.76 | 0.45 |
| Brood size D17 | -0.51 | 0.15 | -0.80 – -0.21 | -3.37 | **0.001** |
| **Random Effects** | | | | | |
| **$\sigma^2$** | 0.34 | **τ00 (nest:colony_id)** | 0.59 | **Observations** | 50 |
| **N (nest)** | 18 | **τ00 (colony_id)** | 0 | **Marginal $R^2$ / Conditional $R^2$** | 0.257 / 0.728 |
| **N (colony_id)** | 3 | **ICC** | 0.63 | | |

*Chick telomere length and apparent survival rate*

We found that sociable weaver chicks with longer rTL at D9 tended to have a higher apparent survival rate the following five years although the 95% credible interval overlapped zero (estimate [95% credible interval] = 0.21 [-0.072, 0.52], p (estimate>0) = 0.928, **Figure 2**). We found no clear association between rTL at D17 and apparent survival (estimate = -0.013 [-0.24, 0.22], p (estimate>0) = 0.458).

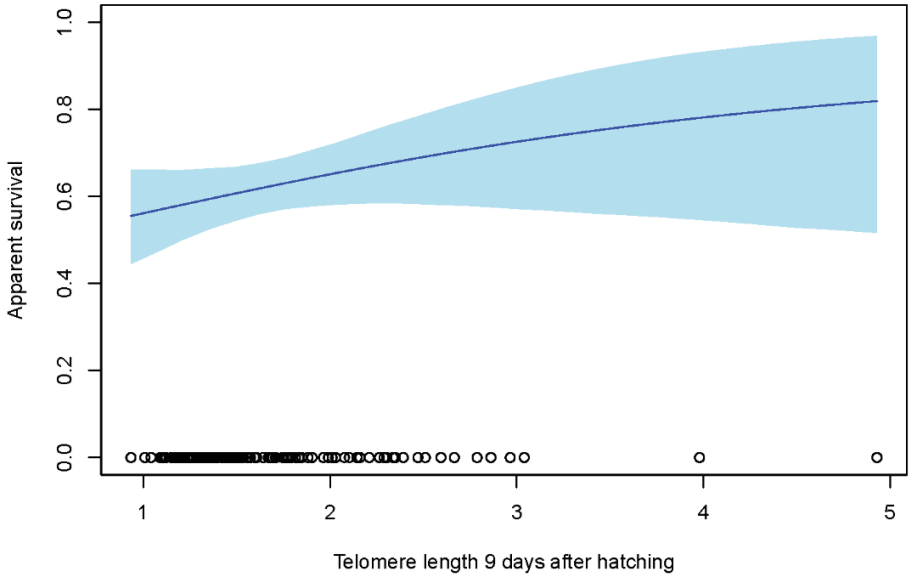


**Figure 2: Relation between relative telomere length (rTL, T/S ratio) 9 days after hatching and apparent survival.** 132 birds were annually captured from 2014 to present (6 years). Estimate and 95% Credibility Interval = 0.21 [-0.072 ; 0.52], p (estimate>0) = 0.928.

**Discussion**

We investigated the influence of the prenatal and postnatal presence of helpers on rTL and the link between rTL and survival in nestlings of the sociable weaver, a cooperatively breeding bird. Early-life rTL (*i.e.* measured nine days after hatching) was overall positively associated with the presence of post-natal helpers but was also influenced by an interaction of the presence of pre- and post-natal helpers. Specifically, we found that for chicks coming from nests without prenatal helpers, rTL was longer when the number of postnatal helpers increased. For the chicks coming from nests that already had prenatal helpers, telomere length was shorter when helpers were reduced after hatching. For those chicks however, an increase in the number of postnatal helpers had no significant relationship with offspring rTL. The interaction between the prenatal presence and postnatal helper change showed that the increase in postnatal helpers was associated to a higher offspring rTL when no prenatal helper was present. The effects observed nine days after hatching (D9) were not observed at the end of the nestling period (D17; but see below) .Finally, the CMR analysis conducted to assess the link between early rTL and post-fledgling survival showed a tendency for rTL at D9 to be positively related to survival up to five years after hatching, but this relationship was no longer present at D17.

*Contrasting effects of pre- and post-natal helpers on offspring's telomeres*

Chicks from nests without helpers had longer rTL at D9 when raised with helpers than without. On the other hand, reducing the number of postnatal helpers in nests that had prenatal helpers was associated with shorter rTL. These two findings support a beneficial effect of post hatching helpers on offspring rTL in sociable weaver. Such positive postnatal influence is thought to be mediated by the enhancement of developmental conditions

that nestlings experience through the additional care provided (Covas *et al.* 2008). This additional care may also contribute to decreasing sibling competition, which has been shown as a mechanism underlying rTL in chicks from different species, either directly (Nettle *et al.* 2015) or indirectly (Voillemot *et al.* 2012; Boonekamp *et al.* 2014; Young *et al.* 2017). A negative effect of sibling competition on offspring development was also suggested here through a negative effect of brood size on body mass. Nevertheless, this beneficial effect appears to be limited, since the addition of postnatal helpers in nests already harbouring prenatal helpers had no significant effect.

We found in addition an interaction between the prenatal presence and postnatal helper change indicating that the positive effect of postnatal helpers is partly dependent on the presence of prenatal helpers. Indeed, when the number of helpers were added after hatching, chicks with prenatal helpers had an average shorter rTL than those without prenatal helpers; the effect persisted even when removing the two extreme values for the 0/1 condition (**Figure 1A**). This outcome is expected if there is a negative prenatal effect resulting from a reduced maternal investment during egg production in presence of prenatal helpers. In fact, previous studies have shown that conditions experienced during embryonic life may altered telomere length at birth (Entringer *et al.* 2011; Haussmann *et al.* 2012; Vedder *et al.* 2018). Sociable weavers females were found to lay smaller eggs with lower steroid hormone content (Paquet *et al.* 2013). Telomere shortening may thus arise from smaller eggs with lower nutritional reserves / less favourable maternal hormone deposit for the embryo development in the presence of pre-hatching helpers.

A non-exclusive explanation for the lack of a positive effect of an increased number of postnatal helpers in nests with prenatal helpers might be that, above an optimal threshold, adding more helpers would have no benefits, or could even become detrimental for offspring rTL. A previous study in the same species has actually shown that increasing group size may result in a weaker offspring survival likelihood (Covas *et al.* 2011, but see Wood 2017 chap. 3). It may also be kept in mind that the positive effect of helpers on chicks after hatching is heightened in difficult environmental conditions (Covas *et al.* 2008). If fewer helpers provide sufficient help under usual climatic conditions, this may explain why we found no significant effect of an increased number of helpers post-hatch when prenatal helpers were already present (1/+1 vs 1/0 and 1/-1).

Given the association between helpers' presence and rTL at day 9, it may be surprising to find no association at day 17. During the time elapsed between 9-days old and fledging, the nestlings experience diverse conditions, such as increased sibling competition (Nettle *et al.* 2015) environmental conditions, and even changes in group size (Ferreira 2015), which could further influence rTL. In addition, the difference in the way our samples were

obtained at day 9 and before fledging preclude us from drawing strong conclusions about the relationship between helper presence and rTL at fledging. Additional data are therefore needed to provide a conclusive test of whether prenatal effects have a relatively limited duration in time and of the role of additional key factors in shaping fledglings' rTL in our species.

*Body mass growth, telomeres, and survival*

Our results are in accordance with the hypothesis that rTL at D9 is associated with survival rate over five years after hatching. The idea that offspring's early rTL is a proxy of survival rate has been found in other species (Geiger *et al.* 2012; Heidinger *et al.* 2012; Stier *et al.* 2014; Young *et al.* 2017; Eastwood *et al.* 2019; Chatelain *et al.* 2020); but see (Boonekamp et al. 2014) or (Wood and Young 2019) for a critical view on telomere shortening and survival). Our study also suggests that chick rTL early after hatching may predict post-fledging survival, at least up to 5 years after hatching. Heidinger et al. (2012) have shown that rTL measured within the first month in zebra finches predicted lifespan better than subsequent measurements. In line with this finding, the lack of association between rTL and survival that we found for older chicks (just before fledging) might suggest that the earlier the measure is, the better the prediction. Moreover, the relationship between individual survival and early-life telomere length are complex, and its significance may vary with age and species (Ingles and Deakin 2016; Lieshout *et al.* 2019).

While here the presence of helpers after hatching is related to longer rTL at D9, and longer rTL is positively related to higher survival rate after hatching, this effect is apparently in contradiction with a previous study that found a negative relationship between helper presence and post-fledging survival (Covas *et al.* 2011). However, this latter result may arise from a later fledging date when the nestlings are fed by larger groups, which increases the duration of nestling's exposure to predation by snakes (Ferreira 2015). Therefore, the environmental and social contexts in which growth is conducted need to be studied in more detail (including proper repeated sampling at different stages of growth) to understand their causal links with rTL and survival.

The improved rearing conditions induced by postnatal helpers could have led also to improve body condition. However, and similarly to a previous study in the same species (Paquet *et al.* 2013), both mass at D9 and fledglings' body condition (D17) did not change with the presence of helpers. These results support previous suggestions on this and other species in showing the ability of helpers to compensate for the lower maternal investment in eggs so that, on average, fledglings have the same body condition. Since, in our study, chicks varied in their day 9 rTL but not body condition, it seems that for the chicks that do not have helpers allocation of energy is toward growth at the expense of their telomeres. Such a trade-off between growth and telomere

maintenance has already been shown in numerous vertebrate species (Tarry-Adkins *et al.* 2009; Geiger *et al.* 2012; Stier *et al.* 2014; Pauliny *et al.* 2015; Ringsby *et al.* 2015; Vedder *et al.* 2017, 2018). A quicker post-hatching growth ensures a higher success rate at fledging (Hipfner and Gaston; Kersten and Brenninkmeijer 1995; Harris *et al.* 2008; Canestrari *et al.* 2011; Loock *et al.* 2017) and an earlier fledging date may decrease predation risk in sociable weavers (Ferreira 2015). As a result, there could be a stronger evolutionary pressure on body growth rather than telomere length; in other words, short-term survival by avoiding predation rather than long-term survival through somatic maintenance. Alternatively, resource-investment trade-offs may be reflected not in body condition or mass, but in tissue maturation rate, which could be reflected in shorter telomeres (Criscuolo *et al.* 2019).

*Conclusion*

Here, we set to investigate the effect of the social environment (presence of helpers) before and after hatching on offspring rTL. Our results suggest an overall positive effect of postnatal helpers on the offspring rTL early in life, but our study did not allow us to conclude that this relationship is conserved until fledging. Furthermore, the positive postnatal effect of helper presence was dependent on prenatal conditions, being stronger in nests that did not have pre-natal helpers. Thus, the presence of helpers may induce opposite effects depending on whether helpers are present before or after hatching and that the interaction between the prenatal and postnatal environment is essential to understand the consequences of the social environment on offspring. In addition, according to our results, the chicks with the shortest telomeres in their early life may also be less likely to survive the first years of life. Although other factors influence rTL, such as genetics or the quality of care provided, our study suggests that social context, in interaction with the ecological context, has a potential important role to play in influencing individual survival in cooperatively breeding birds. The presence of helpers in the early social environment of Alpine marmots (*Marmota marmota*) has also been associated with a longer offspring life expectancy (Berger *et al.* 2015), suggesting an evolutionary convergence between mammal and bird cooperative breeders. The mechanisms that sustain those early social effects on rTL remain to be identified and further studies on the underlying molecular mechanisms (*e.g.* hormones, oxidative stress, metabolic pathways) in chicks, before and after hatching, are now needed.

**Author contributions**

BF, CD, MP and RC contributed to the conception and design of the study. CD, FT, MP and RC collected the data in the field. CD, FT, MP, MQ and RC organised the database. MQ performed the statistical analysis and MP the survival analysis. MQ wrote the first draft of the manuscript. CD, FC, MP, RC and SZ wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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**Data availability:** Until the article is published, dataset and other electronic supplementary materials are available at https://ncloud2.zaclys.com/index.php/s/nLAdQ8Qrm43aAfi > Chapter 2

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**Conflict of interest:** None

# Conclusion | Part 1

## Telomeres confirmed as witnesses of beneficial and detrimental effects of social environment in birds

These first two chapters confirm the reliability of telomere length (TL) to reflect the social environment in birds. Indeed, as well in a species with hierarchical ranks such as zebra finches, as in a cooperative breeder such as sociable weaver, each time TL varied according to the social parameter, highlighting both negative and positive consequences. These two chapters also provide a better understanding of the causes and consequences of telomere shortening, in relation to survival and oxidative stress. Except in some rare studies (e.g. Boonekamp *et al.* 2017), the causal link between oxidative stress and telomere length *in vivo* is often assumed without being tested (but see Reichert and Stier 2017). The strength of the study proposed in **Chapter 1** lies in the measure of multiple variables under controlled conditions. It thereby quantified the contribution of telomere attrition attributable to oxidative stress *in vivo*. Furthermore, this first chapter, which focus on the harmful influence of conspecific aggression in zebra finches, has described a chain of causality, both behavioural and physiological, rarely demonstrated in a single study. While the first chapter focuses more on the causes of telomere shortening, **Chapter 2** illustrates its consequences on the chicks of a cooperative breeder bird, the sociable weaver.

In this and other cooperative breeder birds, it was already known that the helpers contribute to the reduction of the energy cost of parental care. However, few studies had focused on the influence of helpers on offspring phenotype and none had explored the link with their survival rate. Our study first showed a generally positive effect of helpers on chicks' telomere length when measured nine days after hatching. Still, this positive post-hatching social influence is mitigated by the presence of helpers at the nest before hatching. These longer telomeres nine days after hatching were translated into a higher survival likelihood in chicks five years after fledging. Establishing the link between the presence of helpers, telomere length and the survival of chicks in a wild population of cooperative breeders advantageously complements our knowledge of the benefits and costs of a social structure with cooperative breeding. Together with previous studies showing the benefits of helpers for parental survival and reproduction, our results further enlighten why cooperative breeding species in birds live longer than non-cooperative breeders. Still, our study could be improved in several ways. Firstly, continuing to monitor individuals over a longer period will provide a better understanding of the relationship between the presence of helpers, telomeres and individual fitness. In addition, future analyses of individual variation in telomere length should be extended to the whole social weaver study

population, in order to address heritability-related questions, heritability being a factor, likely socially and environmentally dependent, and suspected to prime individual telomere length dynamics over life (e.g. Slagboom *et al.* 1994; Olsson *et al.* 2011; Asghar *et al.* 2015). Finally, conducting this follow-up again on a regular basis will also make it possible to test the influence of climatic conditions on population telomere length. It is likely that the effect of helpers on offspring phenotype may be magnified when seasonal conditions are bad.

Social organisations of zebra finches and social weavers greatly differ, from mainly antagonistic interactions within a gregarious group of individuals to a cooperative breeding system. Still, the large gradient of animal sociality extends up to eusocial systems, in which co-evolution of long- and short-lived related individuals has been magnified. In that case, we need to evaluate how ageing pathways have been specifically modulated. In social insects, societies are organised in caste of specialised individuals with a very specific social role (*e.g.* male, queen, nest-workers, soldiers, foragers). This social role, the caste, controls the rate of ageing (e.g. Chapuisat and Keller 2002; Hartmann and Heinze 2003; Amdam *et al.* 2005; Moroń *et al.* 2008). That said, depending on the species of social insects, the task specialisation can be more or less strict. Thus, even within eusocial species, a gradient in the level of sociality can be observed, with some species strongly eusocial ( characterised by worker sterility, strong caste dimorphism, a single queen monopolises reproduction) and others less so (few or no morphological differences between queens and workers, several reproductive individuals in the colony). The next chapter aims at understanding whether difference in sociality can induce different physiological responses, while controlling for the influence of phylogeny, as well as the biological and ecological characteristics of the species.

- Part 2-

# Challenging the classical biomarkers of ageing in social insects

# Chapter 3| Soma maintenance theories of ageing in the light of ants' lifespan diversity

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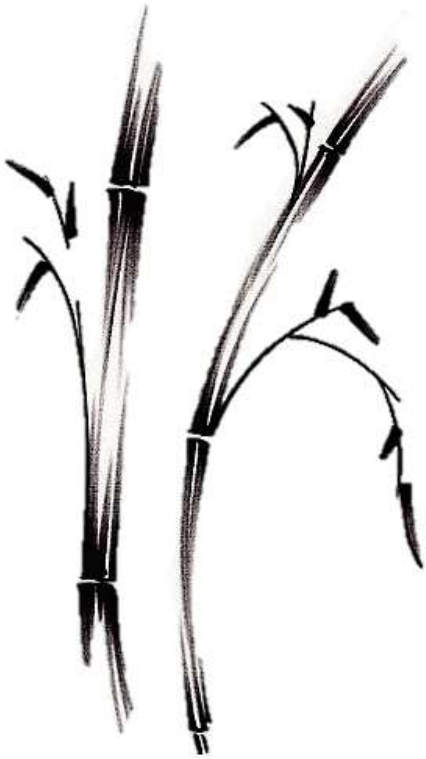
Sur la pointe d'une herbe
Devant l'infini du ciel
Une fourmi

Takarai Kikaku



On the tip of a grass blade
Facing the infinity of the sky
An ant

Takarai Kikaku

## Foreword: The long walk to qPCR amplification in ants

*Take up the methodological challenge of telomere length measurement in 10 ant species using quantitative real-time amplification (qPCR).*

To measure telomere length in biological samples, two main methods are used (Nussey *et al.* 2014). A first, historical one, is the Terminal Restriction Fragments (TRF) measurement by Southern blot. This method consists of using an artificial DNA sequence, complementary to the telomeric sequence of the target species (*i.e.* a probe), which is labelled (with a radioactive or fluorescent marker) and thus reveals the length of the telomeric sequences. The longer the telomeres are, the more repeated sequences, the more probes are attached and therefore the higher is the fluorescent signal (see **Figure S1**). The main advantage of this method is to provide an absolute and accurate value (in number of base pairs) of telomere length.

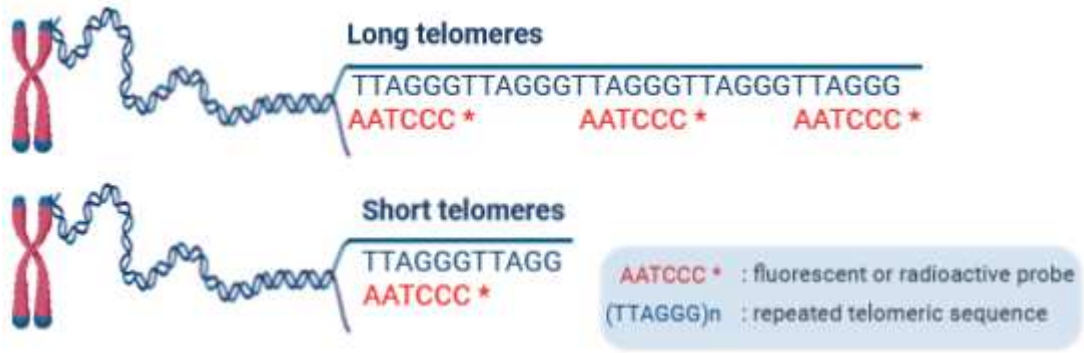



**Figure S1. Schematic view of the telomere length measurement by TRF method.** The longer the telomeres, the stronger the signal when revealed on an electrophoresis gel by Southern blot. (Made with BioRender)

A second method is the measurement by quantitative polymerase chain reaction (qPCR). PCR is a reaction developed by Karry Mullis, who received the Nobel Prize of Chemistry in 1993 for this achievement. PCR amplifies a DNA fragment a very large number of times. To do this, the two DNA strands are separated by heating at 94-96°C. Lowering the temperature to around 65°C allows artificial sequences called primers and specific to the targeted sequence to hybridise with the sample DNA. Finally, the temperature is raised up to *ca* 72°C, the optimal temperature for the activity of Taq polymerase, the enzyme responsible for extending the DNA sequence from the previously hybridised primer. These three steps (denaturation, annealing and elongation) constitute a PCR cycle. It takes several cycles for the fluorescent nucleotides integrated into the newly synthesised sequence to give a detectable signal. This number of cycles before the detectability threshold (called Cq or Ct or sometimes Cp) depends on the efficiency of the polymerase reaction and the size of the sequence to be amplified. The larger the sequence, the more fluorescent nucleotides will be integrated in one cycle, and therefore the less cycles will be needed to obtain a detectable signal. In order to have a reliable value of telomere

length, the measurement is made with reference to another gene, known as the control gene, the size and copy number of which do not vary between the samples being compared. This is mainly due to the fact that the quantity of DNA present in the reactive mix will obviously affect the Cq values (*i.e.* the more DNA, the smaller the Cq). The Cq of one sample for the telomeres is compared with that of the reference gene, and thereby normalised for the DNA quantity. This relative value gives the telomere length of a sample according to a detailed calculation that takes into account the qPCR efficiencies of the qCR reactions (see Pfaffl 2001). This method therefore does not give the absolute telomere length, but rather a relative telomere length using a ratio Telomere / Control gene amplification). The values obtained cannot therefore be directly compared between different studies. However, the qPCR allows a large number of samples to be processed with high sensitivity. To our knowledge, the measurement of telomere length in a social insect, especially in ants, by qPCR has never been published. As our aim was to compare telomere lengths in 10 different ant species, we therefore had to set-up an original protocol by: (i) finding a similar control gene conserved in all our ant's species, (ii) designing primers for the control gene and telomere sequence that produced comparable amplification efficiencies using unique amplification conditions, (iii) finding a statistical method that allows normalisation of the relative telomere lengths and thereby to compare them between species.

*Primer design*

As we were carrying out our study in several distinct species, the sequence of the control gene needed to be highly conserved over the chosen species and not to vary in size or copy number. The sequence therefore had to be not too specific to be present in all the species studied, but still sufficiently to amplify only the control gene and not another portion of the genome. Several candidate genes were evaluated: *gapdh*, *wingless*, *ultrabithorax*, *long wavelength rhodopsin*, and *abdominal homeobox protein A*. Only the gene coding for the long wavelength rhodopsin (LWRh) met all the criteria: same copy number (single copy), gene sequenced and sufficiently conserved in all the target species. The percentage of identity matrix between LWRh sequences, calculated from clustal omega, showed: i) for the whole sequence (ca. 500 bp): min = 73.89 %, max = 93.1%, mean = 79.98 %; ii) for the amplicon (101 bp): min = 84.16%, max = 96.04 %, mean = 91.08 %. The most unsimilar sequences came from *Platytyrhea punctata*. When blast against ants (NCBI taxid:36668), the primers only matched with the target gene. Below is the alignment of the consensus sequence and that of the species studied (**Figure S2**). When designing the primers, we also considered general guidance about qPCR amplification, especially about primer length (18-25 bp), Tm (55-65°C with a maximal difference between both primers of 2°C), %GC (40-60, optimal = 50%, with as few as possible guanine repeats), amplicon length (100-300 bp, e.g. Bustin and Huggett 2017; Thornton and Basu 2011). Once the sequence of primers was chosen, it was necessary to

ensure that the primers did not self-amplify (self-dimer) or bind to each other (cross-dimer) and that the general biochemical characteristics of the primers allowed their proper amplification[6]. The primers used to amplify telomeric sequences in vertebrates cannot be used in Hymenoptera, as the repeated pattern differ (TTAGGG and TTAGG respectively, see Frydrychová *et al.* 2004 for a phylogenetic distribution of telomeric repeats in insects). However, Britt Heindinger's team (Department of Biological Science, North Dakota State University) was kind enough to communicate us the sequences they had designed for the honeybee. I take advantage of these lines to thank them warmly once again.

*DNA extraction*

The extraction protocol finally used in this study is the result of a long phase of gradual improvements of an initial protocol (adapted from Hunt 1997), through numerous modifications throughout the study. In summary, the initial protocol indicated: **a)** grind the animals under liquid nitrogen; **b)** incubate in 500 µL of lysis buffer (0.75 M NaCl, 50 mM Tris/HCl (pH 8), 10 mM EDTA, 1% CTAB) at 65°C; **c)** overnight incubation at 55°C with 2 µL of proteinase K; **d)** add 500 µL of a chloroform-isoamyl alcohol solution (24:1); **e)** vortex and centrifuge (5min, 14 000 rpm, room temperature); **f)** place the supernatant in a fresh tube and adding 40 µL of sodium acetate (pH 4.8) and 350 µL of isopropanol (-20°C); **g)** incubate at least one hour (-20°C); **h)** remove the supernatant, wash with ethanol, centrifuge 5 min at 14 000 rpm. Carry out this operation three times in total, varying the percentage and temperature of ethanol (100% -20°C, 70% -20°C, 70% RT) and the centrifugation temperature (4°C, 4°C, RT); **i)** allow the tube to dry after the last wash and solubilise the DNA in 40 µL of ultrapure water or TE buffer (pH 8).

For good quality DNA extraction, a narrow single peak at 260 nm on the absorbance profile is expected, the wavelength of maximum absorbance for pure nucleic acids. A nucleic acid solution may be contaminated by proteins not removed during the purification steps (here chloroform washing and addition of CTAB to the lysis buffer) or by solvents and salts not removed when the DNA is precipitated and washed (here isopropanol precipitation and ethanol washing). Proteins have an absorbance peak at 280 nm and solvents at 230 nm. In addition to the general appearance of the profile, absorbance ratios at those different wavelengths can therefore be used to assess the DNA quality. Low 260/230 or 260/280 ratios (< 1.8) are indicative of excessive solvent and protein contamination, respectively.

[6] We controlled those biochemical features with online tools available at:
https://www.thermofisher.com/fr/fr/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html
https://www.eurofinsgenomics.eu/en/ecom/tools/oligo-analysis/
http://www.bioinformatics.org/sms2/pcr_primer_stats.html

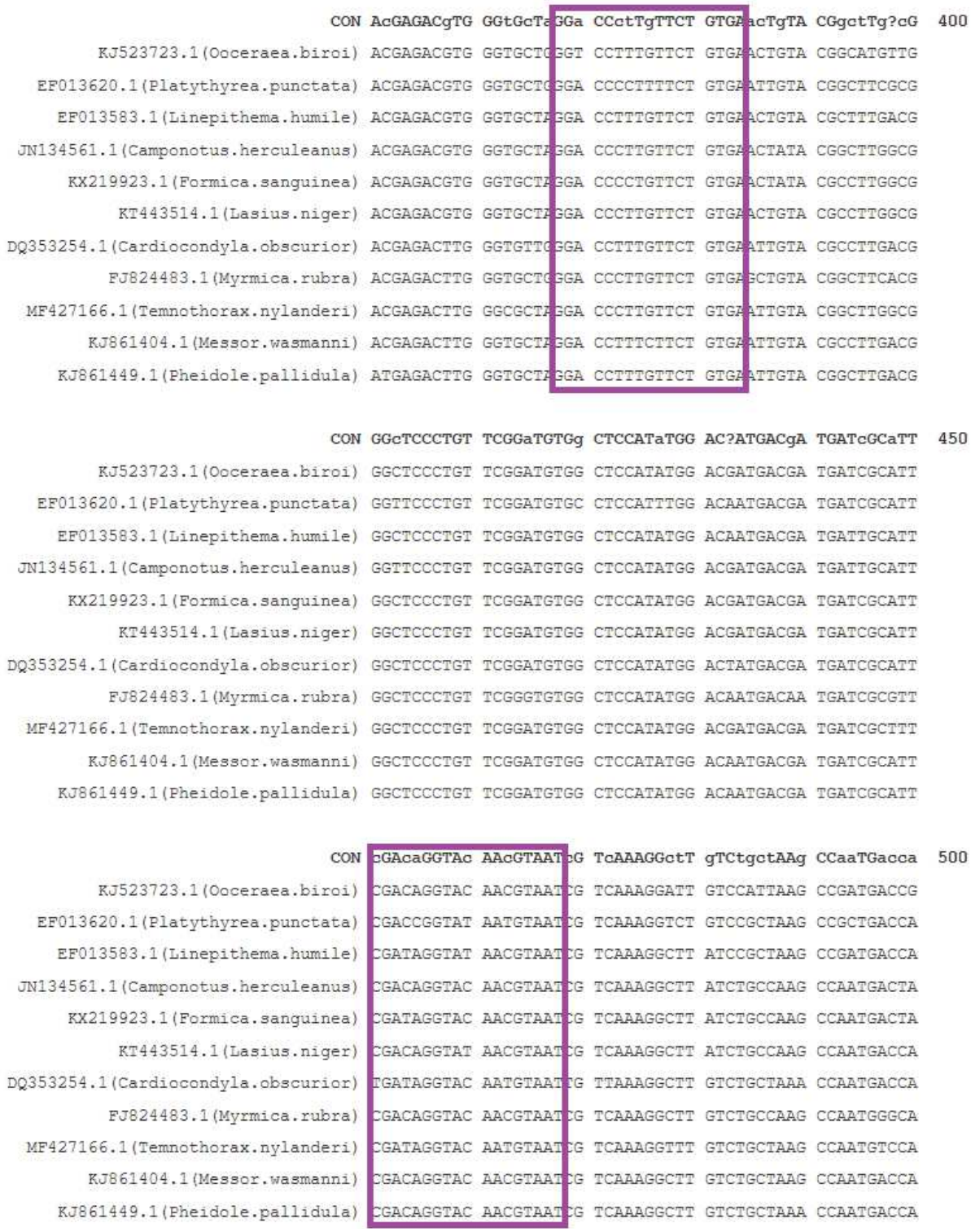



**Figure S2. Sequence alignment of the LWRh gene.** On the far left are the NCBI identifiers of the sequence and the species name in brackets. At the top and in bold is the consensus sequence. An uppercase letter means that this nucleotide is identical in all species, otherwise the nucleotide is lower case. The first and second purple boxes indicate the location of the forward and reverse primers, respectively. The amplified sequence therefore consists of the primers plus the base pairs between them. Alignment made with clustal omega https://www.ebi.ac.uk/Tools/msa/clustalo/.

In the case of these first extractions (**Figure S3.1**) the initial protocol led to average values of DNA concentration and 260/280 ratio that could be improved (44.51 ng/µL and 1.79 respectively) and the average 260/230 ratio was very poor (0.92).

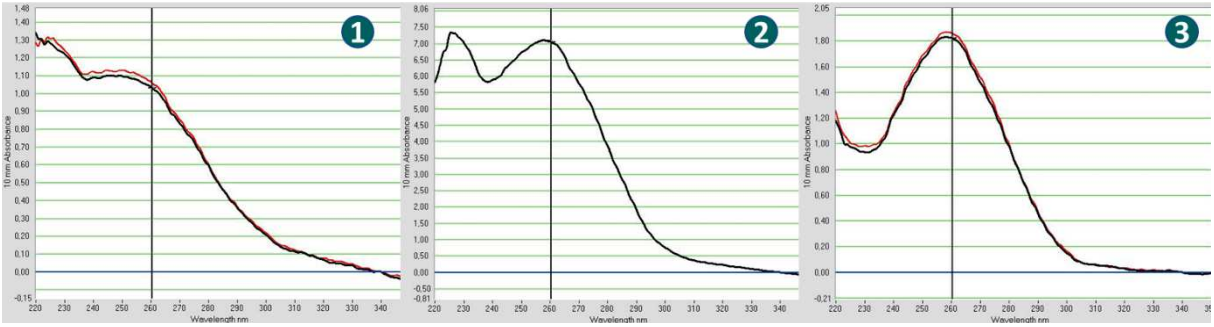



**Figure S3. Typical absorance spectra of samples depending on the protocol used.** Two curves drawn on the same plot means that the measurement was done in duplicate. The following values of DNA concentration and ratios at 230, 260 and 280 nm are averaged ± SEM for all samples (both queen and worker samples) done with the concerned protocol. ① No use of phenol-chloroform-isoamyl alcohol solution (PCI): [DNA] = 44.51 ± 8.88 ng/µL, 260/280 = 1.79 ± 0.09, 260/230 = 0.92 ± 0.10 ② PCI step added + DNA precipitation without salts: [DNA] = 157.76 ± 78.96 ng/µL, 260/280 = 1.84 ± 0.03, 260/230 = 1.35 ± 0.18 ③ modified lysis buffer (CTAB 0.5%, PVP 2%, NaCl 2M, b-ME 1%) + PCI step + DNA precipitation 24h with 100% ethanol and ammonium acetate: [DNA] = 113.33 ng/µL ± 18.02, 260/280 = 1.96 ± 0.01, 260/230 = 2.29 ± 0.05.

It was therefore necessary to accentuate the purification step. For this purpose, before washing the samples with the chloroform solution, we added a step of precipitation of proteins and lipids using a solution of phenol-chloroform-isoamyl alcohol (PCI, 25:24:1) followed by centrifugation (5 min, 14,000 RPM, RT). The supernatant from this step is then washed with the chloroform solution to remove phenol residues and the last protein and/or lipid contaminants. In addition, we carried out the DNA precipitation with salt-free isopropanol to limit the sources of contaminants. As the quality of the extraction was improved both in terms of DNA concentration, 260/230 and 260/280 ratio, we wanted to test whether this was sufficient for PCR amplification. We obtained amplifications of the *LWRh* gene where the lower the 260/230 ratio, the more the number of cycles increased, regardless of the 260/280 ratio or the initial DNA concentration (**Table S1**). This indicated either that the contaminants were inhibiting the PCR reaction or that these contaminants were interfering with the reading of the DNA

| | [DNA] (ng/µL) | 260/280 | 260/230 | Ct |
|---|---|---|---|---|
| **1.** | 92.125 | 1.86 | 1.92 | 24.72 |
| **2.** | 104.77 | 1.91 | 1.455 | 25.82 |
| **3.** | 67.875 | 1.835 | 1.46 | 25.92 |
| **4.** | 471.5 | 1.83 | 1.05 | 29.6 |
| **5.** | 52.55 | 1.75 | 0.855 | 31.1 |

**Table S1. Five samples of different DNA purity.** The five samples below have been chosen for their differences in DNA concentration and ratios. We recorded the number of amplification cycles needed to detect a signal (Ct). We can see a strong negative association between Ct and absorbance ratio 260/230. The higher the Ct, the lower the initial quantity of DNA or the stronger the inhibition of PCR.

concentration at the nanodrop, leading us to overestimate the initial quantity and therefore to overdilute the sample before amplification.

As the salts during the isopropanol precipitation had been removed, the source of contamination did not come from them. We then tested the different solvents used (PCI solution, chloroform alone, CTAB) to see which had an absorption profile that could match the one observed.

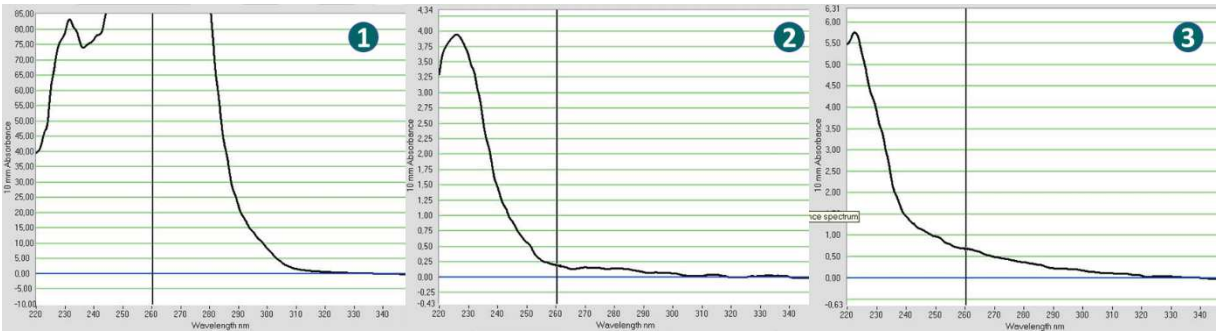



Figure S4. Absorance spectra of solvents used for ant DNA extraction. ① phenol-chloroform-isoamyl alcohol solution (PCI, 25:24:1): [DNA] = 4898,55 ng/µL, 260/280 = 1.11, 260/230 = 1.24 ② Hexadecyltrimethylammoniumbromid (CTAB, 1%): [DNA] = 9.69 ng/µL, 260/280 = 1.41, 260/230 = 0.05 ③ chloroform-isoamyl alcohol (24:1): [DNA] = 34.27 ng/µL, 260/280 = 1.86, 260/230 = 0.18.

Both the profile of CTAB (**Figure S4.2**) and chloroform alone (Figure **S4.3)** could correspond to the extra peak observed at 220-230 nm (**Figure S3-1.2**). Furthermore, both and especially chloroform absorb at 280 nm and could therefore lead to an overestimation of the amount of DNA actually present in solution. To fix potential chloroform-related issues, we paid even more attention not to pipette the slightest drop when recovering the aqueous phase after centrifugation. For potential CTAB-related issues, we redesigned the protocol from the very first steps to limit its possible co-precipitation with DNA. The concentration of CTAB in the lysis buffer was halved and that of NaCl was raised to 2M to make the polysaccharides removal as effective as possible (Fang *et al.* 1992). After overnight incubation with proteinase K, we added a centrifugation step (15 min, 15 000 g, 8°C). Centrifugation is done cold because CTAB precipitates below 15°C (Surzycki 2000, chap. 1, p7), but not too cold so as not to precipitate DNA too early. After that, no more steps are done cold precisely so as not to precipitate residual CTAB. Finally, we have increased the number of ethanol washes up to 5 successive ones if the final pellet is particularly heavy.

Due to the large number of washes and the addition of cleaning with a PCI solution, the possibilities for DNA loss are numerous. We therefore sought to recover the maximum amount of DNA during the incubation phases of the protocol: 15-hour incubation with proteinase K, precipitation with absolute ethanol and ammonium acetate salts for 24 hours at room temperature (Crouse and Amorese 1987).

In addition, the ant cuticle is rich in phenolic compounds absorbing at 230 nm (Andersen 1979; Lockey 1988). To get rid of them, we adapted protocols used to extract DNA from plants, since plants have also a lot of phenolic compounds (Doyle 1991). The new lysis buffer was therefore composed as follows: 2M NaCl, 50 mM Tris/HCl (pH = 8.0), 10 mM EDTA, 0.5% w/v CTAB, 2% w/v polyvinyl pyrrolidone (PVP). And just before incubation at 65°C, we added beta mercaptoethanol (b-ME, 1% v/v). With this modified protocol, the overall ratios were much better, and the DNA concentrations was largely sufficient for use in qPCR (see **Figure S3.3** above).

*qPCR amplifications and statistics*

Conditions of amplifications, encompassing temperature and duration of annealing and extension steps have been first set-up on *Lasius niger* samples (both queens and workers to check for caste-specific putative differences) and then extended to other ant species. The ultimate check points of equivalent qPCR amplifications among species were **(i)** a similar value of efficiencies of the qPCR reaction of the control gene that should stand between 90 – 110% (100% being a doubling of the initial quantity of DNA at each qPCR cycle) and **(ii)** an equivalent size of the amplicons (*i.e.* DNA fragment amplified to an expected size referring to primers sequences). All of these data are presented in the Electronic Supplementary Material of the following article (See **Chapter 3 – ESM2**). Since we ended with still a significant variance in the amplification of the control gene among species (Cq values varying between 23 to 37 cycles), we created 5 classes of species relatively to their control Cq values (23-25, 26-28, 29-31; 32-34, 35-37) and used each of them as a single qPCR run to calculate individual relative telomere lengths within each runs, Then, following an improvement method for telomere comparisons among qPCR-based telomere studies (Verhulst 2020), we used intra-run scaled values of individual relative telomere lengths to further study the evolution of telomere length – longevities in ants.

## Short communication: Mechanisms of Ageing and Development

### SOMA MAINTENANCE THEORIES OF AGEING IN THE LIGHT OF ANTS' LIFESPAN DIVERSITY

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**Abstract**

We aimed at determining (i) the contribution of oxidative stress and telomere length as underlying ageing mechanisms to the maximal lifespan potential (MLSP) of ants and (ii) how telomere length (TL) has coevolved among caste and species of different levels of sociality. Individuals of 10 ant's species, queens and workers, were used to extract body homogenate (with gaster removed) telomere length and oxidative balance markers: non-enzymatic (OXY) and enzymatic (superoxide dismutase) antioxidant activities, and oxidative damage (8-oxo-dG). We found that interspecific variance in MLSP and TL has a low phylogenetic contribution suggesting that MLSP and TL has evolved differently even in closely related species. Our analysis also suggests that high sociality, low oxidative stress, and body size have significant positive effects on queen MLSP but are associated with short TL (high sociality). While diapause is associated with long TL.

**Keywords:** Oxidative stress, telomere, maximal lifespan, social insects, phylogeny, social castes, social organisation (or eusociality)

Eusocial insects are recognised as tremendous models to study the evolution of ageing (Jemielity *et al.* 2007). Twenty years ago, a seminal paper on ageing in ants questioned the evolutionary origins of the extraordinary long lifespan in queens that has co-evolved with eusociality in ants (Keller and Genoud 1997). The decrease in extrinsic mortality risks (*i.e.* predation) of sheltered queens was proposed as the major factor driving such a co-evolution (Lucas and Keller 2020), with variables such as the reproductive strategies (monogyny *vs.* polygyny) and the associated patterns of colony founding (independent – without helping workers- *vs.* dependent) being highly and positively correlated with queen lifespan particularly in ants (Keller and Genoud 1997; Keller 1998). However, the selection of underlying anti-ageing mechanisms in social insects remained uncovered at that stage, and further studies explored the ageing pathways proposed by the free radical theory of ageing (Harman 1955) such as antioxidant capacities (Parker *et al.* 2004; Lucas and Keller 2018) or DNA damage (Lucas *et al.* 2017a), the role of DNA-protein complexes (telomeres) involved in cell senescence(Jemielity *et al.* 2007; Korandová and Frydrychová 2016) or of hormones regulating the trade-off between development, fertility and longevity (Keller and Jemielity 2006). However, to date no interspecific comparisons of such (equivocal) mechanisms in the determination of ant species lifespan or in the interspecific variation of telomere length have been conducted. Furthermore, such an analysis was never extended to ant workers, in order to test whether sociality may have also modulated their much shorter lifespans.

The present study addresses those caveats by investigating whether DNA damage (8-oxo-dG), antioxidants (superoxide dismutase (SOD), whole antioxidant capacity (OXY) and telomere length are related to longevity and sociality both in reproductive and non-reproductive individuals, respectively termed as queens and workers. We measured longevity as the longest life span recorded in a given species, with distinctive values between queens and workers . This measure of longevity is called maximal life span potential (MLSP). The sociality of the 10 ant species was evaluated using a rank calculation based on 3 variables: (i) the difference in body size between queens and workers, (ii) the mating system and (iii) the level of worker sterility. Final ranking was reduced to 3 classes to match with the study sample size (43 individuals). This final ranking is further referred to as 'social index' and reflects whether the division of labour occurred more or less strictly within the considered species.

**Table 1: Construction of the social index.** We summed the scores of each species for three criteria reflecting the strength of division of labour: difference in queen and worker body size, mating system, and worker sterility. This sum ('*sum of scores*') forms the primary social index that we had to split into three categories because of small sample size to end up with the '*final score*', referred to in the main text as 'social index'.

| Species | Body size | | | | Mating System | | Worker sterility | | | Sum of scores | Final score |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Q | W | Ratio Q/W | score | class | score | original class | simplified class | score | | |
| *Camponotus herculeanus* | 17 | 9 | 1.941 | 4 | monogynous | 3 | in absence of queen | mid | 2 | 9 | **2** |
| *Formica sanguinea* | 10 | 8 | 1.333 | 2 | mixed | 2 | in absence of queen | mid | 2 | 6 | **1** |
| *Lasius niger* | 9 | 4 | 2.125 | 4 | monogynous | 3 | rare production of workers thelytokously | mid | 2 | 9 | **2** |
| *Linepithema humile* | 12 | 6 | 1.917 | 3 | not monogynous | 1 | sterile | strong | 3 | 7 | **1** |
| *Myrmica rubra* | 7 | 4 | 1.625 | 3 | mixed | 2 | in presence and absence of queen | weak | 1 | 6 | **1** |
| *Messor wasmanni* | 12 | 7 | 1.643 | 3 | monogynous | 3 | Thelytoky in close species in absence of queens | mid | 2 | 8 | **2** |
| *Ooceraea biroi* | 2 | 2 | 1.000 | 1 | not monogynous | 1 | fertile | weak | 1 | 3 | **0** |
| *Pheidole pallidula* | 8 | 3 | 2.500 | 4 | mixed | 2 | sterile | strong | 3 | 9 | **2** |
| *Platythyrea punctata* | 6 | 7 | 0.910 | 1 | not monogynous | 1 | in absence of queen | mid | 2 | 4 | **0** |
| *Temnothorax nylanderi* | 4 | 3 | 1.414 | 2 | monogynous | 3 | in presence of queen | mid | 2 | 7 | **1** |

We have measured oxidative status and telomere length in ten species of ants while only one species (*Lasius niger*) has been studied so far. Our analysis also gather data about ant morphology (body size and mass) and life history traits (*e.g.* diapause, MLSP, mating system) that can be used in future studies in close or distant research fields, available in Electronic Supplementary Material **(ESM1).** Finally, we provide a reliable experimental protocol for the measurement of TL by quantitative polymerase chain reaction (qPCR). This is the reason why we believe that this study will not only bring new knowledge of the ageing mechanisms but will also pave the way for new studies in this field which is still poorly explored.

The choice of species was done to cover a broad phylogenetic spectrum (**Fig. 1**) and social organisations found in ants, detailed in ESM1, each species having a known sequence of a control gene (**ESM2**) compatible with telomere qPCR amplification (**Table ESM2**). All the species were bred in captivity and whole bodies of 43 pooled individuals (18 queen and 25 worker samples, **Table ESM2**) from 10 species were conserved intact at -80°C before oxidative stress or telomere measurements. For simplicity, we call '*queens*' the individuals, the main function of which is reproduction at the time of collection. Conversely, the term '*workers*' refers to samples containing individuals, the main function of which is not reproduction at the time of collection (*e.g.* brood caring, foraging). The characteristics of these individuals may vary according to the species considered (see **ESM1**). Samples were prepared as followed: gasters were removed on dry ice and upper bodies (head, thorax, and legs) were pooled to reach at least 10 mg before grinding (with the exceptions of *Pheidole pallidula* queen (5.93 mg), *Platythyrea punctata* workers (4.27 mg) and *Temnothorax nylanderi* queen (3.20 mg). Pools of upper bodies were ground under liquid nitrogen in a 1.5 mL Eppendorf tube with a plastic pestle and 600 µL of PBS were added to the homogenate. Then, these 600 µL were apportioned between DNA extraction for TL measurement (300 µL), oxidative stress markers (200 µL) and protein concentration (100 µL). Full protocol of DNA extraction is given in **ESM3**. Individual telomere length was assessed using qPCR method producing a relative telomere length expressed as Telomere / Control amplifications (T/S ratio, (Cawthon 2002) and adapted for ant as detailed in **ESM2**. Oxidative balance markers were measured following the manufacturer's recommendations, *i.e.* DNA damage (8-oxo-dG, ELISA Kit, StressMarq Biosciences, Victoria, Canada), total superoxide dismutase activity (SOD, Enzo Life Sciences, Inc., Farmingdale, NY, USA) and whole antioxidant capacity (OXY, OXY-Adsorbent test, DIACRON Labs, Grosseto, Italy). Oxidative balance measurements were performed during a unique session on samples always kept on ice and all measurements were performed in duplicate.

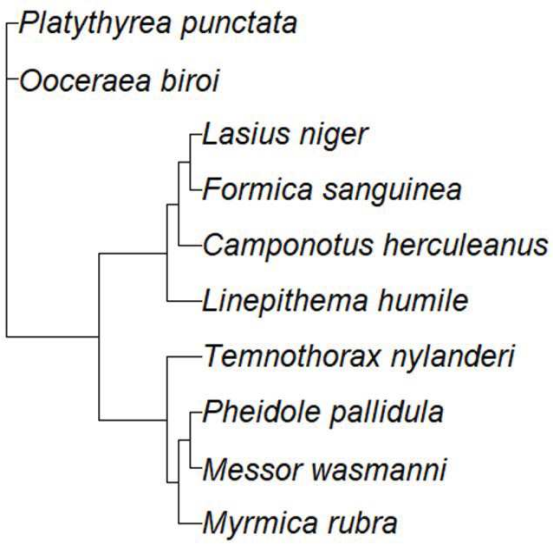


**Figure 1:** Phylogenetic tree of 10 ant species based from the sequence alignment of the long wavelength rhodopsin (LW-Rh) gene, obtained using Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/). *LW-Rh* gene sequences are available is ESM2. Tree was constructed using *apTreeshape* and *caper* R package.

All data were scaled before analysis (dataset of mean centred values for each variable, **ESM2**), which was of particularly importance for comparisons of qPCR relative TL values among species (Verhulst 2020). Statistical analysis was done using R version 3.5.1 (R Foundation for statistical Computing Platform, 2018) following three steps: (i) principal component analysis (PCA) was run to reduce the number of physiological variables, creating 2 PCA axes that will be used as fixed factors to explain MLSP variance: PCA1, the body mass dependent oxidative balance axis, positively loaded Oxy and negatively loaded with DNA damage and body mass and PCA2, the telomere and antioxidant axis, positively loaded with SOD and negatively with TL (detailed in **ESM4**) (ii) evaluation of the covariance among MLSP and TL due to shared ancestry of species by computing a phylogenetic generalised least square models using the R package caper (*pgls*; Orme *et al.* 2018). *Pgls* were run using means values *per* species of all variables, queens and workers being analysed separately (ESM3). In both cases, the $\lambda$ parameter estimating the phylogenetic influence was 0, indicating that MLSP and TL variation were independent of phylogeny in our dataset (**ESM4**); (ii) consequently, the determinants of MLSP and TL were estimated using the 43 pooled samples and separated Linear mixed models (LMM) with *Species* as random factor, and *Caste* (queen, worker), *Sociality* (**ESM4**), *Diapause*, *PCA1* and *PCA2* using *Mumin* R package for model selection (Bartoń 2013).

MLSP was found to be significantly influenced by the interaction Sociality x PCA1, but differently in queens and workers (Caste x Sociality x PCA1 interaction, **Table 1**). While MLSP was not dependent of PCA1 in workers, queens of highly social species were long-lived (**Fig. 2A**) and presented lower DNA damage levels and higher antioxidant capacity (**Fig. 2B**). TL appeared to be affected by Diapause and Sociality but in a Caste dependent manner (Interaction Caste x Diapause and Caste x Sociality, Table 2): queens from species with diapauses in their life cycles had significantly longer telomeres (**Fig. 3A**) while those of high sociality tended to have shorter telomeres (**Fig. 3B**).Our data underlined that MLSP is influenced by sociality in ants in a caste dependent manner, queens of species characterised by a marked

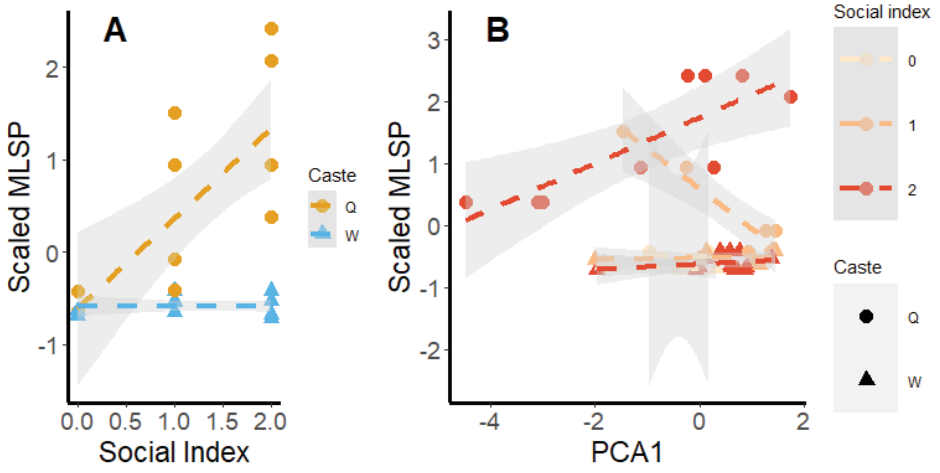


**Figure 2: Ants' maximal lifespan potential (MLSP)** in relation to **(A)** species level of eusociality (Social index) and **(B)** PCA1 (representing oxidative balance in relation to body mass, ESM4) measured in queens (Q, n=18) and workers (W, n=25) caste and in 10 different species. Confidence interval at 95% are indicated in grey around the regression lines. Note that the regression of queens with a social index of 1 and 2 were significant (see ESM3).

social organisation (*i.e.* monogynous mating system, large size difference with sterile workers) being long-lived. Interestingly, this longer lifespan was not totally independent of body mass (small queens living longer) and of oxidative balance (higher antioxidant buffering capacities and lower levels of DNA damage being associated with high MLSP). On the same time, those highly social queens presented also smaller telomeres, while queens of species having diapause had longer telomeres.

Table 1: Linear mixed model testing for the effect of caste (workers and queens), sociality and PCA axes of ant species physiological variables (see ESM4) on their maximal lifespan potential (MLSP). The model is based on 43 observations of 10 ant species encompassing 18 queens and 25 workers pooled samples (see Table ESM2). Species was used as random factor to control for repeated measurements within species (queen and workers of each species were measured in duplicates, ESM2). Significant effects ($P \leq 0.05$) are indicated in bold and model selection was done using AICc values (*Mumin* R package). Random effects are described using $\sigma^2$ and $T_{00}$ statistics calculating the intraclass correlation coefficient of the random factor (ICC). $N_{id}$ indicate the number of levels for the random factor (*i.e.* number of species). Marginal $R^2$ expressed the total variance explained by the selected model only including the fixed factors while the Conditional $R^2$ takes fixed and random factors into account.

**MLSP of ants (Months)**

| Predictors | Estimates | std. Error | CI95% | Statistic | p |
|---|---|---|---|---|---|
| Intercept | -0.33 | 0.24 | -0.80 – 0.13 | -1.4 | 0.161 |
| Caste (workers) | -0.31 | 0.22 | -0.73 – 0.11 | -1.43 | 0.152 |
| Sociality | 1.02 | 0.16 | 0.71 – 1.32 | 6.45 | **<0.001** |
| PCA1 | -0.97 | 0.25 | -1.45 – -0.48 | -3.87 | **<0.001** |
| Caste x PCA1 | 1.17 | 0.33 | 0.53 – 1.81 | 3.59 | **<0.001** |
| Caste x Socialiy | -1.01 | 0.14 | -1.29 – -0.74 | -7.23 | **<0.001** |
| Sociality x PCA1 | 0.61 | 0.13 | 0.35 – 0.86 | 4.61 | **<0.001** |
| Caste x Sociality x PCA1 | -0.67 | 0.2 | -1.06 – -0.28 | -3.37 | **0.001** |
| *Marginal $R^2$ / Conditional $R^2$* | *0.821 / 0.905* | *$\sigma^2$ = 0.08* | *τ00 Species = 0.08* | | |

Table 2: Linear mixed model testing for the effect of caste (workers and queens), sociality and Diapause of ant on their relative telomere length (expressed as scaled values of T/S ratio). Data used encompasses 43 observations of 10 ant species (18 queens and 25 workers pooled samples, see Table ESM2). Species was used as random factor. Significant effects ($P \leq 0.05$) are indicated in bold and model selection was done using AICc values (*Mumin* R package). Description of random effects is given in Table 1.

**Telomere length of ants (Scaled T/S ratio)**

| Predictors | Estimates | std. Error | CI95% | Statistic | p |
|---|---|---|---|---|---|
| Intercept | -0.67 | 0.29 | -1.24 – -0.10 | -2.32 | **0.02** |
| Caste | 0 | 0.26 | -0.51 – 0.51 | 0 | 0.997 |
| Diapause | 0.93 | 0.49 | -0.04 – 1.90 | 1.89 | 0.059 |
| Sociality | 0.52 | 0.3 | -0.07 – 1.11 | 1.74 | 0.083 |
| Caste x Diapause | -0.88 | 0.43 | -1.72 – -0.03 | -2.03 | **0.042** |
| Caste x Sociality | -0.5 | 0.26 | -1.00 – 0.00 | -1.95 | 0.052 |
| *Marginal R2 / Conditional R2* | *0.712 / 0.847* | *$\sigma^2$ = 0.14* | *τ00 Species = 0.13* | | |

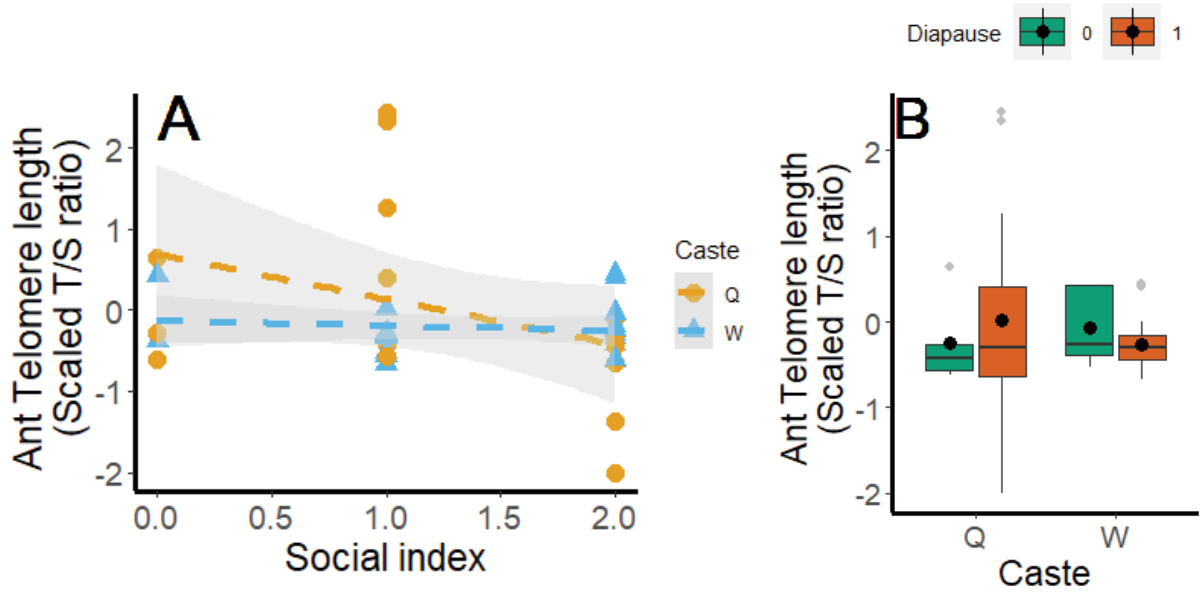


**Figure 3**: Ants' relative telomere length (scaled T/S ratio) in relation to **(A)** species eusociality level (Social index) and **(B)** diapause event during the species life cycle (0 = none, 1 = present), measured in queens (Q, n=18) and workers (W, n=25) caste and in 10 different species. Boxplots indicate minimum and maximum, median (black line), first and third quartile and mean (black dots on the boxes) values. Confidence intervals 95% are in grey. Note that none of the regressions were significant (see ESM4).

Our results have different implications for our understanding on the evolution of ageing in social animals. First, eusociality, *via* the reduction of the risk of extrinsic mortality is predicted to be associated with increased longevity due to an increased investment in somatic maintenance (Kirkwood *et al.* 1979). While this hypothesis had obtained mixed supports so far, particularly in ants (Lucas and Keller 2014), our data nevertheless confirmed that queen's lifespan may be determined by their DNA maintenance (Lucas *et al.* 2016) or antioxidant buffering capacities. However, this link between oxidative balance and lifespan was found here to be highly dependent of sociality, suggesting that predictions of the oxidative stress theory of ageing should be re-evaluated (at the interspecific level and not only between queens and workers) by accounting better for social effects. It may be that lifespan differences between social castes among ants have differently evolved among a gradient of social organisations (Lucas and Keller 2020). Second, while we found no evidence for a telomere based MLSP evolution in our study, (long-lived) eusocial queens are still characterised by short telomeres. In addition to the correlative association between diapause (*i.e.* periods of reduced metabolism) open an interesting and hypothetical parallel with our knowledge of telomere evolution in hibernating mammals, which also repair their telomeres during periods of hypo-metabolism (Wang *et al.* 2011; Turbill *et al.* 2012, 2013) , and in rodents s(Tian *et al.* 2018). In short, social rodents have long telomeres and use other cell mechanisms to protect themselves from the ever-long telomere associated risk for lifespan: the cell immortalization and cancer (Seluanov *et al.* 2018). Since we found that sociality has co-evolved with small telomeres and with long-

lifespan, but that telomeres were not associated directly to ants' MLSP, our data rather suggest that short telomeres and high MLSP have indirectly co-evolved because of social related factors, but do not have direct causal relationships.

Phylogenetic constraints on ants' MLSP and telomere length were found to be negligible in our study, highlighting that they have evolved differently in closely related species. However, both were highly influenced by the social characteristics of the species. The phylogenetics approach, using 10 different species of ants, brings confounding factors that could unfortunately not all be perfectly handled. The first is undoubtedly age. The workers were each time pooled for analysis, grouping older and younger individuals lightened the potential effect of age. The queens were all at sexual maturity and not at the end of their lives, but the age could not be harmonised more precisely between species. Some species came from worker castes with dimorphisms (*minor* and *major* castes). Special care was taken to ensure that the samples contained an equivalent mass of minors and majors when this was the case. This allowed us to prevent variations between worker samples within the same species, but it may have led to a loss of information. For example, minor workers live longer than the major workers in the ant *Oecophylla smaragdina* (Chapuisat and Keller 2002). Sexual or asexual (clonal) reproduction was partly taken into account in the social index, but this variable might be worth testing separately as it is a major difference in the life history traits of a species.Finally, some ants were facultative polyandrous species (Boomsma and Van Der Have 1998), which means that several males might have fertilised the queens and thus brought more genetic diversity than in monoandrous or clonal species. However, we did not know if mutiple fertilizations took place or not. Those possible counfounding factors call for more comprehensive experimental studies on local adaptations and on the different cell pathways implicated in ageing and potentially modulated by social interactions.

**Authorship**

MQ, FC and CS contributed to the conception and design of the study, MQ acquired the data under the supervision of SZ for the telomere length, MQ and FC contributed to the analysis and interpretation of the data, MQ and FC drafted the manuscript, further revised by CS and SZ. MQ, FC, CS and SZ approved the final draft.

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# Conclusion | Part 2

## An innovative study that discusses the mechanisms of ageing across the tree of life

The present study is characterised, to our knowledge, by several innovative aspects, from the methodologies used to compare physiological and social parameters that may be implicated in longevity evolution among (a curtailed limited) ant phylogenetic tree, to the social conditioning of the implication of diapause, telomere length or oxidative stress in queens' maximal lifespan.

*Methodological innovations*

The first aspect of broad interest of our study is the development of **(i)** a rigorous DNA extraction protocol and **(ii)** of an adapted oligonucleotide primer allowing the measurement of telomere length using qPCR in ten species of ants. Previously, the analysis of telomere length was limited to caste comparisons within a specific species, and to a few rare species: the honey bee *Apis mellifera* (Korandová and Frydrychová 2016), the bumble bee *Bombus terrestris* (Koubová *et al.* 2019), and the black garden ant *Lasius niger* (Jemielity *et al.* 2007). In a single study, we have therefore provided precious additional data to the current knowledge on co-variation in telomere length or oxidative stress with lifespan in the different social castes. In addition, using an index of eusociality (applicable to most social insects), we highlighted that for instance, oxidative balance may be more implicated in queens' lifespan among species than previously believed based on intra-specific studies.

*Impact of sociality and well-known ageing mechanisms*

Thanks to our index of the eusociality level, we were able to study the role of eusociality in the modulation of ageing mechanisms. We first confirmed that MLSP was positively associated to the degree of eusociality. Second, we highlighted that sociality (reflected here by two aspects, caste and the eusocial continuum) modulates the implication of some mechanisms defined as lifespan determinants in other taxa. In fact, only the queen features (MLSP; telomere length and even more surprisingly the oxidative balance) were affected by sociality. In addition, these associations were found more marked in the queens of strictly eusocial species. Sociality thus act both directly by expanding MLSP, but also indirectly by modulating the ageing mechanisms depending on the caste. Still, the causality between telomere length and MLSP was not demonstrated here and needs further dedicated experiments.

Long-lived and eusocial queens were characterised here by shorter telomeres. The lack of the classical positive association between telomere length and longevity has also been observed in long-lived mammals – more especially rodents. Among these species, telomere length maintenance does not

correlate with life expectancy but rather with body size (Seluanov *et al.* 2008, 2018; Tian *et al.* 2018). Shorter telomeres in ant queens might act as an overall anticipated protective mechanism, limiting the risk of cell immortalization in reproductive adults. This appears to be a plausible explanation even in ants, since cancer has been described in insects and will represent a risk in those long-lived (over decades) ant eusocial queens (Aktipis *et al.* 2015).

*The diapause effect: a conserved mechanisms of telomere maintenance and longevity evolution? About the importance of studying telomerase activity.*

Hibernating vertebrates live longer than non-hibernating ones, and present an interesting ability to restore their telomeres during hypometabolic (Turbill *et al.* 2011; Constant *et al.* 2020) periods. The beneficial effect of diapause on telomeres found here suggests that there could be a convergence of life-history traits and mechanisms in ant species using diapauses and hibernating mammals (Wang *et al.* 2011; Turbill *et al.* 2012, 2013). In another social insect, the bumblebee (*Bombus terrestris*), queens were characterised by an increase in telomerase activity before diapause, confirming a potential diapause-related mechanism of telomere lengthening in queens of social Hymenoptera (Koubová *et al.* 2019). Again, we based our discussion on correlative data, and we cannot exclude additional explanations. For instance, compared to those living a shorter life, small long-lived rodents, repress telomerase activity and use additional protective mechanisms that may sustain longer lifespan such as tumour suppressor mechanisms (Seluanov *et al.* 2008, 2018; Tian *et al.* 2018). This may also be the case in our long-lived ant queens. The analysis of telomerase activity would allow us to answer such questions, by comparing the parallel evolution of anti-cancer systems in small long lived but distant social taxa. In ants, previous studies tend to show a higher activity of telomerase in queens compared to workers (Jemielity *et al.* 2007; Korandová and Frydrychová 2016; Koubová *et al.* 2019). However, we saw that MLSP and telomere length could depend on the degree of sociality of the species considered, and on the diapause. We thus need to control for these parameters to have a more comprehensive view of telomerase regulation and consequences within the diversity of insect taxa. It was initially planned to carry out a telomerase activity measurement on the 10 ant species as well. There are still few published studies exploring telomerase expression levels in social insects and the direct application to another species is not easy. We had started to develop a protocol in ants, but the design of the necessary oligonucleotides raised technical issues. Unfortunately, the end of the PhD and the sanitary context did not allow us to complete the trials we had started.

*Multiple improvements at the intra- and inter-specific scales to better understand the ageing-sociality nexus*

In order to address more comprehensively the impact of caste on the mechanisms of ageing, we could refine the scale of our study by differentiating the caste of workers. In honey bees and weaver ants, it is known that foragers age more rapidly than nest-workers, regardless of the chronological age (Chapuisat and Keller 2002; Amdam *et al.* 2005; Baker *et al.* 2012). Would the acceleration of ageing observed during caste change be reflected by the mechanisms we have studied here? If so, this could explain why we have not observed any effect of the variables studied on MLSP and telomere length in workers: the effect of different castes blurring each other. Although males are reproductive individuals like queens, eusociality did not co-evolve in them with increased longevity. In the vast majority of social bees, ants, and wasps, males live even shorter than workers: a life span restricted to the breeding season. In addition, hormone secretions differ significantly between males and females, particularly the quantity (Trenczek *et al.* 1989; Gätschenberger *et al.* 2012) and timing of expression during development (Piulachs *et al.* 2003) of vitellogenin, which plays a key role in the differentiation of female castes. It is therefore to be expected that, although belonging to the same species, the mechanisms of ageing are radically different in this caste. The rare data that exist on the comparison of males and females in terms of senescence point towards this direction, since in ants, only the males have telomere sizes in line with their lifespan (Jemielity *et al.* 2007). According to the late deleterious mutation theory of ageing and because of their very short life, males are expected not to have evolved effective antioxidant defence mechanisms, which could explain their shorter telomeres. However, differences in the mechanisms of ageing between males and reproductive or non-reproductive females could be due to differences in sex (hormonal context), reproductive status, MLSP, or behaviour. An ant genus of choice to decipher this multifactorial impact of male caste on their ageing mechanisms might be *Cardiocondyla*, which provide both female and male castes differing in fertility, behaviour and longevity (Schrader *et al.* 2015). Indeed, the colonies of this genus host ergatoid (wingless) males remaining in the nest and living longer than winged males, potentially because they benefit from a lower extrinsic mortality risk (Heinze *et al.* 1998).

If our study suggested that the degree of eusociality appeared to interact with the ageing mechanisms in reproductive individuals, additional data collected alongside the ant eusociality gradient are needed to strengthen these general description pattern. For example, ponerine ants are phylogenetically primitive ants with no or weak age-dependent caste shift (i.e. age polyethism; Traniello 1978) and a flexible reproductive division of labour. In fact, queen castes does not really exist since the reproductive individuals, called gamergates, come from the worker (sometimes clonal) caste (Peeters 1991). However, the way a worker becomes a gamergate is highly variable (Schmidt and Shattuck 2014). Since our study only used one ponerine ant species (*Platytyrhea punctata*), moreover clonal, we lack statistical power to conclude about how ageing is specifically modulated in primitive ant species. In

addition, our study also underrepresented non-diapausing species. So, including additional species will advantageously complete our understanding of how social variables (clonal reproduction, diapause) actually affect longevity diversity and evolution in ants.

Finally, long-term experiments are necessary to better define the links between telomere length and lifespan in eusocial insects. Unfortunately, their generally small body size is not adapted to carry repeated sampling on the same individual at different ages or life stages. Nonetheless, cross-sectional monitoring remains feasible. They would provide high quality data to control both caste and age of individuals. However, this implies long-term studies since the queens of the longest-lived species can live for several decades, and disentangling the age from the caste effects on a large number of workers necessitates the multiplication of experimentally manipulated (and recorded for individual behaviour) colonies. Such a protocol will be better adapted to unravel the key ageing mechanisms explaining individual variation in lifespan or ageing rate, by disentangling the effects of age and social role. The next part addresses those questions with three chapters using an exploratory approach based on mass spectrometry analysis of proteins and metabolites.

- Part 3 -

# Exploring the interaction between ageing and division of labour in ants through omics

# Foreword

We saw in **Chapter 3** that the longevity of queens was partly in agreement with the general predictions of the free radical theory of ageing. However, some expectations such as a positive link between telomere length and MLSP was not found either in workers or queens. Undoubtedly, senescence in social insects should be mediated by other biological processes. The aim of the next three chapters was to explore the cell and metabolic mechanisms at work to explain the differences in ageing between castes, *via* proteomics and metabolomics. As it was not possible for technical and temporal reasons to carry out these in-depth studies on the same ten species analysed in chapter 3, we have chosen to focus on only one, well-studied, locally collectable and easily reared: the black garden ant *Lasius niger*.

The next three chapters take advantage of the recent advances in mass spectrometry applied to life sciences. Mass spectrometry technique make it possible to characterise the molecular composition of a biological sample. Compounds are identified by measuring the ratio of their mass (m) and their ionic charge (z), known as the m/z ratio. To do this, the sample is introduced into the mass spectrometer in gaseous, liquid, or solid form depending on the method used. At the source of the mass spectrometer, the compounds to be analysed are ionised and, if necessary, desolvated. The analysis of the analytes continues in the gas phase, with the ions being transmitted *via* an interface to the analyser under the effect of a high vacuum and various electrical voltages. The tandem analyser(s) then separates, isolates, and fragments the analytes (ions) according to their m/z ratio. Finally, the detector transforms the ion detection into an electrical signal and amplifies it for further computer processing. The experimental m/z ratios obtained are compared with the theoretical m/z ratio calculated *in silico* from the information contained in the reference databases. It is then possible to identify and quantify all the metabolites (*i.e.* metabolome: *e.g.* lipids, carbohydrates, vitamins) present in a biological sample (*e.g.* blood, tissue). The amino acid by amino acid determination of a protein sequence also makes it possible to identify and quantify all the proteins (*i.e.* proteome) in a biological sample. These analytical methods deliver a large amount of information which must then be methodically analysed, filtered and organised to understand the biological significance of the results. The bioinformatics tool is then precious to accelerate the reading and interpretation of generally massive data. The possibility of accessing biological functions depends on the completion of the databases used. Is the molecule already described in the reference databases and will it therefore be identified? Then, if identified, have other studies shown its involvement in a particular biological process? On the success of conducting these successive analytical

steps will depend the formulation of reliable biological explanations of the purely physico-chemical dataset initially produced by mass spectrometry.

**Chapter 4** aims at understanding how eusociality can shape the proteome of black garden ants. To do this, we compare the proteomes of queens and workers, but also that of nest-workers with that of foraging workers. **Chapter 5** complements the omics picture by studying the metabolome of the same castes in the same species. Thus, we seek to compare the information provided by these two approaches using the same, duplicated, descriptive approach. Our omics exploration of ageing mechanisms between workers' caste is carried out one step further in **Chapter 6**. In black garden ants, as in most ants' species, switch in the social role among workers (*e.g.* nest-worker *vs.* foragers) is dependent on the age of the individual. We therefore had no way of knowing whether the differences observed between workers in Chapters 4 and 5 were due to age or to the metabolic changes following the switch in social role or both. In Chapter 6, we developed a protocol to discriminate the effects of age and social role on ageing mechanisms in workers. Furthermore, we conducted the study of the proteome and metabolome strictly in parallel: the same samples were analysed by both methods at the same time. This reduced any source of individual or temporal bias in the comparison of these two methods of investigation. Altogether, these three chapters addressed how social role and age influence the metabolic and ageing pathways in a social insect.

# Chapter 4 Division of labour leads to three distinct proteomes in black garden ants (*Lasius niger*)

Sur la pivoine blanche
Netteté
De la fourmi.

Sharp
On the white peony
An ant.

Yosa Buson

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# Division of labour in the black garden ant (*Lasius niger*) leads to three distinct proteomes

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ABSTRACT

Task specialization in social insects leads to striking intra-specific differences in behaviour, morphology, physiology and longevity, but the underlying mechanisms remain not yet fully understood. Adult colonies of black garden ants (*Lasius niger*) have a single queen fertilised by one or a small number of males. The inter-individual genetic variability is thus relatively low, making it easier to focus on the individual molecular differences linked to the division of labour. Mass spectrometry-based proteomics enabled us to highlight which biological functions create the difference between queens, foragers and nest-workers. The proteome of each caste reflected nicely their social role: *e.g.*, reproduction for queens, pesticide resistance for foragers – that are the most exposed to environmental risk factors – and, interestingly, digestion for nest-workers, thus highlighting proteomic profiles differences even among workers. Furthermore, our exploratory approach suggests energy trade-off mechanisms – in connection with the theory of social immunity – that might explain the difference in longevity between queens and workers. This study brings evidence that proteomics is able to highlight the subtle mechanisms of molecular regulation induced by social organisation.

## 1. Introduction

Animal species display different schemes of social organisation – from solitary to eusocial species. Eusociality exists in certain mammals (Burda et al., 2000), crustaceans (Duffy et al., 2000) and insects (Wilson, 1971). The latter include eusocial Hymenoptera (wasps, bees, ants) and termites, the complex social organisation of which is based on division of labour. Each individual belongs to a caste and displays a caste-specific set of behaviours. While the queen's main role is to produce offspring, the task specialization among workers is highly species-dependent and can result in a broad range of sizes and shapes within the same species (Harvell, 1994; Jeanne, 1986; Morton Wheeler, 1908; Seeley, 1986). Castes also differ in terms of longevity, queens reaching a dramatically longer lifespan than workers, living up to ten-fold longer (Keller and Genoud, 1997). The reproductive division of labour leads to a higher concentration of ecdysteroids and vitellogenin in reproductive individuals (Gospocic et al., 2017; Robinson et al., 1991). Intriguingly, hormone concentrations do not reflect only the reproductive status but also the task specialization among workers, particularly the trio con- stituted by Insulin/Insulin-like growth factor Signalling, vitellogenin and juvenile hormone (Azevedo et al., 2011; Corona et al., 2013; Guidugli et al., 2005; Kohlmeier et al., 2018; Libbrecht et al., 2013; Nelson et al., 2007). Thus, resulting from division of labour in eusocial insects, genetically close individuals may nevertheless greatly differ from each other in terms of behaviour, morphology, physiology and longevity.

Genomics and proteomics picture different levels of gene expression (Gygi et al., 1999; Hunt et al., 2010). Studying differences between individuals at the proteome level allows consideration of changes which are likely closer to phenotypic variation than, for instance, gene expression (Baer and Millar, 2016). The study of the molecular basis of social life by genomic tools (*i.e.* sociogenomics) has already led to the identification of numerous genes (Robinson et al., 2005; Sumner, 2006). By contrast, proteomics studies in social insects are less focused on behaviour and social interaction, concern mainly the honeybee biology (but LeBoeuf et al., 2016), and are biased toward larvae rather than adults. In the latter case, the proteome of queen-destined and worker-destined larvae has been shown to differ in protein quantity for the following processes: resistance to oxidative stress, energy production, lipid metabolism, amino acid metabolism, development, protein

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biosynthesis, protein folding and cytoskeleton (Li et al., 2010). Larval mitochondrial (Begna et al., 2011) and larval nuclear (Begna et al., 2012) proteome studies have shown similar results, as well as the comparison at an adult stage of antennal proteome between drones (male bees), workers and queens (Fang et al., 2012).

Black garden ants (*Lasius niger*) combine low genetic variations and a marked division of labour. Even though worker's ovaries are functional (van der Have et al., 1988), the queen monopolizes the reproduction, she is larger and lives far longer than workers (Parker, 2010). These elements appoint black garden ants as a wise choice to stimulate the field of "socioproteomics", *i.e.* to determine the influence of the social role on individual's proteome, and then phenotype. In ants, proteomics has been hitherto used to specifically analyse spermatheca or venom compositions (Malta et al., 2014; Wiese et al., 2006), and protein response to desertic conditions (Willot et al., 2017). With the hypothesis that abundance of proteins specific to given tasks and/or related to longevity and reproduction physiology should differ between the castes, we compared the proteome of black garden ant individuals, with the aim to characterize the differences between queens, foraging workers and non-foraging workers (respectively referred to as foragers and nest-workers).

## 2. Materials and methods

### 2.1. Animal model

The black garden ant (*Lasius niger*, Linnaeus 1758) is a very common species in Western Europe, in urban and rural areas (Sterry, 1997). They are omnivorous and widely known to farm aphids for the honeydew they excrete (Domisthorpe, 1927). In adult colonies, only one queen lays eggs (monogynous species). She is fertilised by one or two males, and very rarely by more (Boomsma and Have, 2002; Fjerdingstad et al., 2002; Fjerdingstad et al., 2004). The queen is 7–9 mm long and has an average lifespan of 20 years, whereas workers are 2.5–5 mm long and live for 3 years on average (Hölldobler and Wilson, 1990). Unlike in other species, black garden ant foragers and nest-workers do not morphologically differ from each other.

The colonies used in this study came from wild newly-mated queens captured on the site "Campus Plaine" at the Université Libre de Bruxelles (50°49′08.4″N 4°23′57.0″E) and tended during two years in lab. We removed the eggs to control that all individuals were 2 years old. Colonies, consisting of only females (queen and worker ants), were housed in IPHC-DEPE (Strasbourg, France) at a temperature of about 25 °C with 50–60% relative humidity and were fed with sugar water (0.3 M) and mealworms once a week. Even though no law regulates the care and use of insects, we applied internal animal welfare policies by minimizing the number of ants used in experiments and by preventing any form of avoidable suffering.

### 2.2. Caste identification

The worker castes differ from each other by their interaction pattern and spatial segregation (Mersch et al., 2013). Active individuals, spending time in the foraging area were identified as foragers. On the other hand, the nest-workers were identified by no move outside the nest and their tendency to form immobile clusters. To stimulate the foraging behaviour, we used a 4-day fast and then placed a high con- centration sugar solution (1 M) in a plastic tray. To ensure an optimal recruitment, we waited for 5 min after the fifth individual came to the food source, then picked up all the forager individuals seen at the food source for one hour. No foraging behaviour was noticed after this period in preliminary tests. We then collected the nest-workers. Ants were anesthetized by cold (0.5–1 min, −20 °C). A pen filled with acrylic ink (Posca®) was used to mark their abdomen, with a different colour according to the caste. When they woke up, the ants were carefully watched for a few minutes. None of them exhibited any sign of aftereffects. They were then put back into their colony where usual food and water were provided. This process was repeated three times, every 48 h, to reduce the number of false positive (starved nest-workers ex- iting from the nest) and false negative (non-recruited or non-captured forager).

### 2.3. Proteomic analysis

#### 2.3.1. Sample preparation

We used 15 colonies, individuals of which were homogeneously distributed among the samples. One sample is made of three queens or ten workers (nest-workers or foragers). We had five samples per caste, except in nest-workers, where only four samples had a sufficiently high protein content to be analysed by mass spectrometry. Frozen ants were ground under liquid nitrogen for 45 s at 30 Hz using a Mixer Mill MM400 (Retsch, Eragny Sur Oise, France), and total proteins were ex- tracted from the resulting powder using 200 µl of extraction buffer (8 M urea, 2 M thiourea, 0.1 M Ammonium Bicarbonate, 1% DTT, protease inhibitors; Sigma-Aldrich, Lyon, France). After sonication on ice (2 × 10 s, 135 W) and centrifugation (2000×g, 2 min) to eliminate cuticle residues, 8 volumes of cold acetone were added to samples that were kept at −20 °C overnight. Precipitated proteins were pelleted by centrifugation (13,500×g, 20 min, 4 °C), and after discarding supernatants, dissolved in Laemmli buffer (10 mM Tris pH 6.8, 1 mM EDTA, 5% β-mercaptoethanol, 5% SDS, 10% glycerol). Samples were centrifuged to eliminate the remaining cuticles (2000*g*, 2 min). Total pro- tein concentrations were determined using the RC-DC Protein Assay kit (Bio-Rad, Hercules, CA, USA). At this stage, a reference sample com- prising equal amounts of all protein extracts was made, to be injected regularly during the whole experiment and thus allow QC-related measurements. 20 µg of proteins from each sample were electrophoresed on SDS- PAGE gels (12% polyacrylamide) for 60 min at 50 V followed by 15 min at 100 V. After protein fixation (50% ethanol, 3% phosphoric acid), gels were stained overnight using colloidal Coomassie Blue. For each lane, five 2 mm bands were excised, and proteins were in-gel digested with trypsin (Promega, Madison, WI, USA; 120 ng/band) at 37 °C overnight after de-staining, reduction (10 mM DTT), alkylation (55 mM iodoace- tamide), and dehydration using a MassPrep station (Micromass, Waters, Milford, MA, USA). Tryptic peptides were extracted using 60% acet- onitrile, 0.1% Formic acid in water for one hour at 450 rpm on an or- bital shaker. The organic solvent was then eliminated using a vacuum centrifuge (SpeedVac, Savant, Thermoscientific, Waltham, MA, USA), and peptides were re-suspended in 90 µl of 1% acetonitrile, 0.1% formic acid in water. A set of reference peptides (iRT kit; Biognosys AG, Schlieren, Switzerland) was finally added to each sample prior to LC- MS/MS analyses.

#### 2.3.2. Nano LC-MS/MS analyses

Samples were analysed on a nanoUPLC-system (nanoAcquity, Waters) coupled to a quadrupole-Orbitrap hybrid mass spectrometer (Q-Exactive Plus, Thermo Scientific, San Jose, CA, USA) using a randomized sequence within block injections. Each block consisted of one biological sample of each group plus the reference sample. To reduce carry-over, two solvent blank injections were included in between each sample. Briefly, one µl of each sample was concentrated/desalted on a Symmetry C18 pre-column (0.18 × 20 mm, 5 µm particle size; Waters) using a mobile phase composed of 99% of solvent A (0.1% formic acid in water) and 1% of solvent B (0.1% formic acid in acetonitrile) at a flow rate of 5 µl/min for 3 min. Afterwards, peptides were eluted using a UPLC separation column (BEH130 C18, 200 mm × 75 µm, 1.7 µm particle size; Waters) maintained at 60 °C with the following gradient: from 1% to 6% B in 30 s, from 6% to 35% B in 59.5 min.

Q-Exactive Plus was operated in positive ion mode with source temperature set to 250 °C and spray voltage to 2.0 kV. Spectra were acquired through automatic switching between full MS and MS/MS scans. Full scan MS spectra (300–1800 $m/z$) were acquired at a resolution of 70,000 at $m/z$ 200 with an automatic gain control (AGC) value set to $3 \times 10^6$ ions, a maximum injection time set to 50 ms, and the lock-mass option being enabled (polysiloxane, 445.12002 $m/z$). Up to 10 of the most intense precursors (with a minimum of 2 charges) per full MS scan were isolated using a 2 $m/z$ window and fragmented using higher energy collisional dissociation (HCD), with normalised collision energy set to 27 eV and dynamic exclusion of already fragmented pre- cursors set to 60 s. MS/MS spectra were acquired at a resolution of 17,500 at $m/z$ 200 with an AGC value set to $1 \times 10^5$ and a maximum injection time set to 100 ms, and the peptide match selection option was turned on. The system was fully controlled by Xcalibur software (v3.0.63; Thermo Fisher Scientific). Peak intensities and retention times of reference peptides were monitored in a daily fashion.

#### 2.3.3. *Protein identification and quantification*

MS raw data processing was performed using MaxQuant (v 1.5.3.30). Peak lists were searched against a UniProtKB-derived protein database created using the MSDA software suite (Carapito et al., 2014). The database contained *LASIUS niger* (TaxID 67767) protein sequences (February 2017; 18,075 sequences) to which sequences of common contaminants were added (247 entries; contaminants.fasta included in MaxQuant). A minimal number of one peptide (unique or razor) was required for protein identification. A maximum number of one missed cleavage and a false discovery rate (FDR) of 1% for both peptide-spectrum matches (minimum length of seven amino acids) and proteins was accepted during identification. From the use of a *Lasius niger* protein database, we identified 57 "un characterised" proteins (~4% of all identified proteins) for which we searched known homologous proteins in the Protostomia clade. This was done by using BLAST searches (FASTA program v36; downloaded from http://fasta.bioch.virginia.edu/fasta_www2/fasta_down.shtml), and only the best hits were retained. To validate this procedure, we automatically extracted orthology annotations and sequence domains of *Lasius niger* uncharacterised proteins and of their homologues from the OrthoDB (Kriventseva et al., 2019) and InterPRO (Mitchell et al., 2019) resources. The relevance of the match between *Lasius niger* uncharacterised proteins and their homologues was then checked manu- ally.
Regarding quantification, data normalisation and protein abun- dance estimation were performed using the MaxLFQ (label-free quantification) option implemented in MaxQuant (Cox et al., 2014) using a "minimal ratio count" of one. "Match between runs" was enabled using a one minute time window after retention time alignment. Only unmodified peptides were considered for quantification (except those for which a modified counterpart was detected) while shared peptides were excluded. All other MaxQuant parameters were set as default. Only proteins quantified with at least two unique peptides and detected in at least three samples in a given caste were kept for further analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository with the dataset identifier PXD006779.
Regarding quality controls, we found that the median coefficient of variation (CV) of retention times and raw intensity of iRT peptides when considering all injections was 0.96% and 22%, respectively. The median CV regarding the raw intensity of all quantified proteins across a repeated analysis of the reference sample was 16%. These different values support the good stability of the nanoLC-MS/MS system during the whole duration of analyses, and good reproducibility of protein abundance determination.

### 2.4. *Protein selection procedure AND PCA*

In total, 2707 proteins were identified, of which 1325 fulfilled the criteria for accurate quantification (see above). This original dataset is available online in Supplementary material (Tables S1–S3). To properly run the PCA (principal component analysis); missing data were inferred by regularized iterative PCA algorithm (missMDA package; (Josse and Husson, 2016). The PCA was performed (FactoMineR, v.1.34; Lê et al., 2008) on all three castes, and also only the two workers castes separate from the queen caste, in order to have a more precise insight into po- tential differences. First, we used PCA as a filter to shrink the number of variables and only retain proteins giving rise to differences between the castes. Only 174 proteins for the three castes and 108 for the workers were strongly correlated to the axes ($p < 0.05$) and faithfully projected ($\cos^2 > 0.8$; Fig. S1C and D). Most of the time, protein databases in insects are unfortunately patchy and based on predicted annotations. This makes an automatic annotation highly prone to misassignment. The protein functions were therefore "manually" attributed by using several database resources (Ortho DB, InterPro, UniProt) that contain annotations for proteins from the Protostomia clade that are homo- logous to *Lasius niger* proteins, as well as results from previous studies in insects (social insects whenever possible) to avoid misleading func- tional annotations. In the case of pleiotropic proteins, we kept all the different functions (proteins indicated in blue in Tables S2 and S3) and did not arbitrarily choose one among the others. Finally, 255 proteins were kept for further analyses (Fig. 1), and they were clustered ac- cording to their functions (categories in Table 1). These functional groups were used as variables to build the axes of the second set of PCAs (Fig. 2), in order to determine which biological functions allow dis- criminating queens from nest-workers from foragers."
Most of the time, proteomics databases in insects are unfortunately patchy and based on predicted annotations. This makes an automatic annotation highly prone to misassignment. The protein functions were therefore "manually" attributed by crossing several databases (Ortho DB, InterPro, UniProt) and actual studies in insects (social insects whenever possible) to avoid misleading functional annotations. In the case of pleiotropic proteins, we kept all the different functions (proteins indicated in blue in Tables S2 and S3) and did not arbitrarily choose one among others. Finally, 255 proteins were kept for further analyses (Fig. 1), and they were clustered according to their functions (cate- gories in Table 1). These functional groups were used as variables to build the axes of the second set of PCAs (Fig. 2), in order to determine which biological functions allow discriminating queens from nest- workers from foragers.

### 2.5. *Statistical tests*

Statistical tests were performed using R software (R Core Team, v3.4, 2017) at the significance threshold $\alpha = 5\%$. To statistically test whether any biological function isolates one caste from another, we used the PCA's coordinates as variables. With them, we performed Kruskal-Wallis (KW) tests (> 2 modalities, heteroscedasticity) and Wilcoxon rank sum test (2 modalities, homoscedasticity). If the KW tests were significant, they were followed by Conover-Iman posthoc test with Bonferroni correction (conover.test v.1.1.5; Alexis Dinno). Homoscedasticity was assessed by Bartlett test.

## 3. Results

### 3.1. *Biological functions splitting the castes according to the PCAs*

In the Supplementary material section, we supply accession numbers, names and relative amounts of the 1325 quantified proteins (Table S1B). Tables S2 and S3 aggregate the 255 retained proteins and their functional category. We identified 35 functional groups (Table 1) ac- cording to our selection procedure.
Regarding the three-caste analyses, the first two dimensions (Fig. 2A) of the PCA explained 82.41% of the variance, which is con- sidered significant (Table S4). Only the first axis statistically separated all castes from each other ($\chi^2KW_{axe1} = 9.926$, $p_{axe1} = 0.01$ and $\chi^2KW_{axe2} = 0.591$, paxe2 = 0.744): forager-nest-worker (t = 1.82,

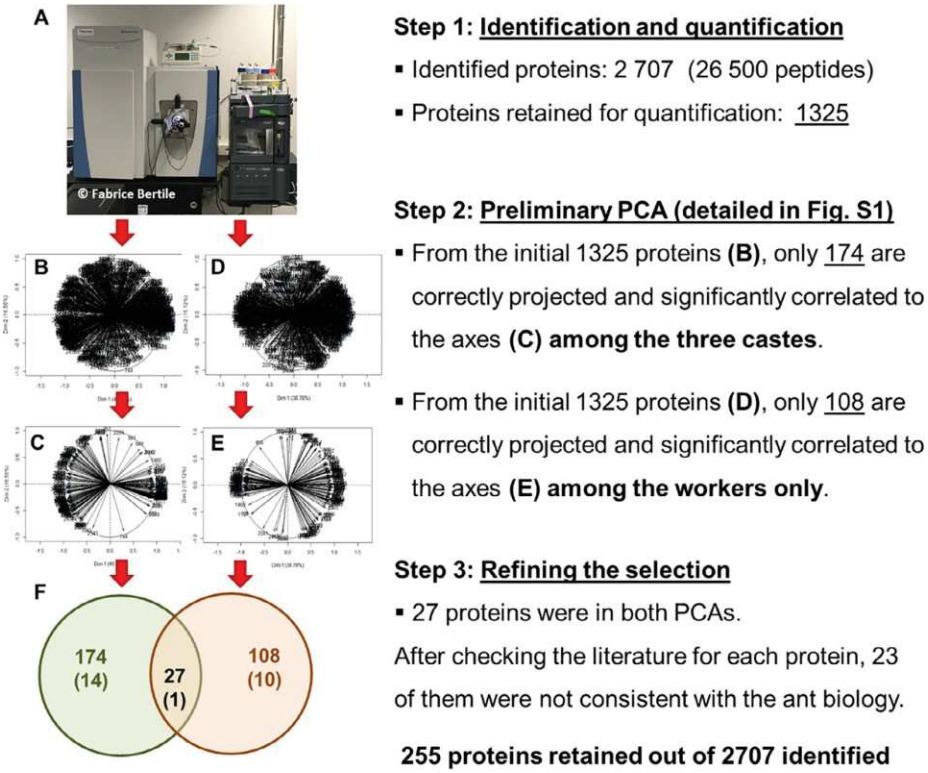


**Fig. 1. Protein selection procedure.** The underlined numbers are the subset in the step n used in the step n+1. A: First, the mass spectrometer and the MaxQuant Software were used to analyse the proteins. Amongst the 2707 identified proteins, 1325 could be quantified. One PCA regarding the three castes (B) and one regarding the workers only (D) were performed with these proteins. Only the proteins properly projected ($cos^2 > 0,8$) and significantly correlated to the axes ($p < 0,05$) were kept for the three castes (C) and the workers only (E). F: The number in brackets refers to the number of inconsistent proteins among the total number. A total of 23 proteins did not show strong evidence to be involved in insect's processes or in evolutionarily conserved mechanisms. 27 proteins were redundant between the two PCAs. The 255 proteins remaining at the end of this procedure were clustered according to their biological function in the further analysis. *Photo credits: FABRICE Bertile, co-author of publication.*

$p = 0.048$), queen-nest-worker ($t = -3.71$, $p = 0.002$), forager-queen ($t = -5.86$, $p < 0.001$). Queens were mainly characterised by proteins involved in somatic maintenance – mechanisms aiming to avoid or repair damages to macromolecules – cell division, gene expression regulation and trafficking. While workers expressed more proteins related to metabolic pathways (except lipid metabolism) and sensitive nervous system or immunity.

In the second PCA (workers only), the first two dimensions (Fig. 2B) explained 87.94% of the variance, which is considered significant (Table S4). The second axis isolated nest-workers from foragers, but the first axis did not ($W_{axe1} = 16$, $p_{axe1} = 0.191$ and $W_{axe2} = 20$, $p_{axe2} = 0.016$). Foragers presented more proteins associated with xenobiotic detoxification mechanisms, whereas nest-workers had more proteins involved in digestion metabolism.

### 3.2. Proteins related to the ToR pathway

The Target of Rapamycin (ToR) protein belongs to the serine/threonine kinase family (Helliwell et al., 1994). A growing literature shows the implication of the ToR pathway in ageing-related diseases (Skike and Galvan, 2018) and its evolutionarily conserved ability to shorten lifespan in various taxa (Powers et al., 2006). We were thus interested in knowing whether the amount of ToR-related proteins would differ between the castes. Four proteins involved in the ToR pathway were significantly different between castes: striatin-3 isoform x2 ($\chi^2_{KW} = 10.73$, $p < 0.001$), peptidyl-prolyl cis-trans isomerase ($\chi^2_{KW} = 9.23$, $p = 0.01$), ubiquilin-1-like isoform 2 protein ($\chi^2_{KW} = 6.17$, $p = 0.04$) and eukaryotic translation initiation factor 4e ($\chi^2_{KW} = 6.94$, $p = 0.03$). Only the cAMP-dependent protein kinase catalytic subunit was not significant ($\chi^2_{KW} = 4.06$, $p = 0.13$). However, they are only marginally related to the ToR pathway and could be involved in other signalling pathways. Hence, we did not perform further analyses.

## 4. Discussion

Our proteomic analysis combined with a PCA-based protein selection highlighted biological functions specific to each caste. The exhaustive list of selected proteins and related functions is available in Supplementary data (Tables S2 and S3). Below, we focus the discussion on some functions (Table 1) or life-history traits that were either different between queens and workers, or, within the workers, between foragers and nest-workers. First, we focus on identified caste-specific functions that define the worker castes. Second, we propose other sources of individual variation amongst the workers. Third, based on observed differences in queens and workers profiles, we discuss possible mechanisms involved in the large difference in longevity. This leads us to question the usual fecundity/lifespan trade-off by considering the energetic cost of an active immune system in a so particular social context.

**Table 1**
**Functional groups retained to build the axes of the PCA.** Functional category's names are adapted from GO term annotations (www.geneontology.org) of proteins homologous to *Lasius niger* proteins, identified using several database resources (Ortho DB, InterPro, UniProt), as well as literature examination. The IDs corresponds to the numbers found on the PCA plots (Figs. 2 and S1). The attribution of proteins to functional groups is detailed in the 'Materials and Methods' section.

| Functional group | ID | Description |
|---|---|---|
| Cell Activity | 1 | Protein involved in several mechanisms highlighting general cell activity: transcription/translation, ATP synthesis… |
| Ageing + | 2a | Direct and/or strong association with the individual ageing status. |
| Ageing - | 2b | Direct and/or strong association with a slower ageing rate or extended lifespan. |
| Apoptosis + | 3a | Inducing or fostering apoptosis. |
| Apoptosis - | 3b | Inhibiting or delaying apoptosis. |
| Tissue Growth | 4 | Tissue growth especially during embryonic development. |
| Chaperone | 5 | Protecting the cell against harmful conditions (oxidative stress, pH variation…). Ensuring a proper protein folding. |
| Cell Cycle | 6 | Controlling cell cycle (mitosis/meiosis, blocking cell cycle…). |
| Cytoskeleton | 7 | Part of the cytoskeleton or associated with (actin, dynein, kinesin…). |
| Detoxification | 8 | Soma repair after a stressful event. |
| Digestion | 9 | Protein involved in the digestion metabolism. |
| Cell Dynamics | 10 | Non-focused action proteins involved in structural cell mechanisms: controlling cell shape, adhesion… |
| GnExpression+ | 11a | Activating gene expression (transcription and/or translation). |
| GnExpression- | 11b | Inhibiting gene expression (transcription and/or translation). |
| Calcium Homeostasis | 12 | Regulating the calcium level. |
| Human Pathologies | 13 | Human diseases – degenerative most of the time (*e.g.* Alzheimer). |
| Xenobiotics Detox | 14 | Resistance to chemicals, especially pesticides. |
| Immunity | 15 | Resistance to pathogens. |
| Larvae | 16 | Proteins related to larval development. |
| NclAcid Metabolism | 17 | Nucleic acids synthesis or modification. |
| Energy Metabolism | 18 | Protein involved at least in one of the following pathways: glycolysis, Krebs cycle, gluconeogenesis, ATP synthesis. |
| Glucid Metabolism | 19 | Glucid modification, not for direct use in glycolysis or Krebs cycle |
| Lipid Metabolism | 20 | Lipid modification, not in an energetic purpose. |
| Protein Metabolism | 21 | Protein synthesis or modification. |
| Muscles | 22 | Protein required for muscle contraction. |
| IR | 23 | Irrelevant: unknown function or inconsistent in *L. niger* |
| Cell Proliferation | 24 | Protein directly involved in cell proliferation. |
| Glucid Recycling | 25 | Breakdown of glucids to provide the cell with new fatty materials. |
| Protein Recycling | 26 | Breakdown of proteins to provide the cell with new amino acids. |
| Redox | 27 | Promoting redox reactions in physiological conditions, ensuring the redox balance within the cell. |
| Reproduction | 28 | Proteins related to the gametes. |
| Secretion | 29 | Secreted proteins: hormones, pheromones, in saliva. |
| Nervous System (NS) | 30 | Growth, maintenance and repair of the nervous system. |
| Sensitive NS | 31 | Protein involved in the sensitive nervous system. |
| Membrane Trafficking | 32 | Protein involved in membrane trafficking between RE and Golgi or related to other transport vesicles (synapses, endo/exocytosis). |

### *4.1. Division of labour has multiple proteomic consequences in workers*

#### *4.1.1. Sensory system*

We found a homologue protein (apd-3-like protein) to the bee's apd3 protein which is, according to a genomic study, related to the olfactive/gustative function in the antennae (Antony et al., 2016). SAP47 was also part of this functional group and is involved in the learning process of smells and pictures in Drosophila's larvae (Saumweber et al., 2011). Ant communication is (almost) all about pheromones. Nest building, foraging, social identification: all rely on those signals (Beckers et al., 1993; Khuong et al., 2016; Yan et al., 2017). Moreover, worker ants must also decipher the environmental cues. For instance, foragers must find the appropriate food sources according to colony needs within an environment full of non-specific odorants. Hence, it is not surprising to see in workers high levels of proteins related to the sensitive nervous system (Fig. 2C), allowing them to detect and analyse those specific and non-specific olfactory cues. This hypothesis is supported by a genomics study in *L. niger*, where workers up-regulate the Ln385_5 gene, involved in odorant binding (Graeff et al., 2007).

#### *4.1.2. Immunity*

Workers had on average a higher amount of proteins associated with the immune system (*e.g.* arginine kinase, T-cell immunomodulatory protein). Some studies have opposing results regarding the expression of immunity-related genes in ants (Graeff et al., 2007) or bees (Grozinger et al., 2007). Moreover, ferritin, known to withhold iron from invading pathogens (Ong et al., 2005) has only been found in queens in our analysis. On the other hand, a study in *Melipona quadrifasciata* (Judice et al., 2006) has found an up-regulation in workers of a gene coding for a scavenger receptor involved in the immune response. The relationship between the caste and the immune system seems hence to be equivocal.

At first glance, the fact that queens are more susceptible to patho- gens because of weak immune defences does not sound evolutionary stable. High productivity in laying eggs is pointless if ant queens do not survive the first encountered pathogen. Moreover, we know that group living makes individuals more prone to infection (Godfrey et al., 2006; Schmid-Hempel, 1998). To overcome these issues, eusocial insects have evolved a combination of behavioural responses, called social immunity (details in Cremer et al., 2007). For instance, infected individuals are less involved in interactions and they can sometimes even be killed by their own colony. Cremer et al. also suggest that the structure of the interaction network might be shaped in a prophylactic way to prevent pathogens from reaching the queen. The queen would be thus "socially" protected from pathogens. In this context, a weaker immune system would not be an inevitably fatal issue. Supporting this assumption, a phylogenetic study in five insect species has shown that the larger the colony size is, the weaker the melanization response (López-Uribe et al., 2016). In addition, ants – similarly to other eusocial insect species – have fewer genes involved in immune functions than less social insects (Libbrecht et al., 2013), what brings evidence that proper social structure can allow for a reduced immune system.

#### *4.1.3. Differences within the worker caste*

Foragers differ from nest-workers by their higher amount of proteins involved in insecticide resistance – mostly cytochrome P450 (CYP). The role of CYP in insecticide detoxification is well documented (Oppert

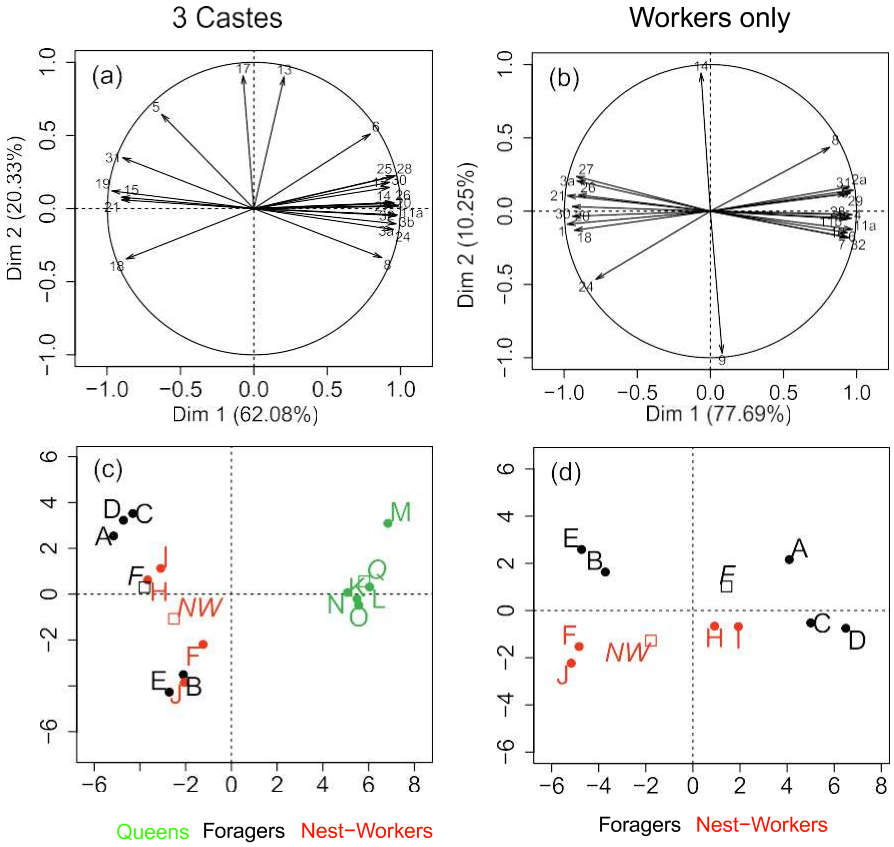


**Fig. 2. PCA amongst the castes of *L. niger*. Left**: charts regarding the three castes. 14 ant pools and 33 variables (biological functions). **Right**: charts regarding the workers only. 9 ant pools and 25 variables (biological functions). (a) and (b): functional groups correlating with at least one of the axes ($p < 0.05$) and with a $\cos^2 > 0.8$, identified by a number (names and functions in Table 1). (c) and (d): representation of the individuals. Similar coordinates mean the individuals show a similar level of protein expression in the same biological functions. The empty squares with a capital letter (Q = queens, NW = nest-workers, F = foragers) indicate the average coordinates for each caste.

et al., 2015; Werck-Reichhart and Feyereisen, 2000). Because of their supplying role, foragers are directly exposed to pesticides. This larger amount of pesticide-degrading proteins may help them to face environmental toxic chemicals. Once detoxified by foragers, the food can be safely distributed to the whole colony. Overexpression of the CYP gene has also been found in the worker caste of *Melipona quadrifasciata* (Judice et al., 2004). This could indicate a common response to insecticides among social insects. A similar response has been observed in honey bee, where the activity of two detoxification enzymes increases when workers begin to forage (Smirle and Winston, 2011). Yet, CYP is also involved in metabolic functions in insects such as hormone degradation (Feyereisen, 1999) and we cannot rule out at that stage other metabolic implications.

We found that a larger amount of digestive proteins (essentially alpha-amylase) are expressed in nest-workers. By storing food excess (Lenoir, 1979) and pre-digesting it, nest-workers can make it quickly available for further use, thus enhancing the fitness of the whole colony by buffering environmental unpredictability in food resources, but also by increasing food processing by conspecifics. Nest-workers may also pre-digest the food for castes that do not perform this task very well. According to several studies, ant larvae do not require help to digest food (Cassill et al., 2005; Erthal et al., 2007; Went et al., 1972). The pre- digested food could be more useful for the queen, allowing her syn- thesizing fewer digestive proteins and saving energy for other costly life history traits (*i.e.* reproduction). Whether this may be of any advantage for other adult castes must be tested by accurately measuring digestive proteins levels of expression in nest-workers and foragers.

### 4.2. *Other sources of variation within the workers*

Social castes appeared to be of major importance to explain the proteome variability among adult individuals in the black garden ant. Nevertheless, unexplained variance remained (Fig. 2C and D) and nest- workers and foragers were not all perfectly collocated, suggesting other sources of individual variation. Tan et al. (2017) highlighted that dia- pause can affect the proteome of the cabbage beetle (*Colaphellus bowringi*). All the colonies were nonetheless under the same day light and temperature conditions, it is hence unlikely that some individuals enter diapause and others did not.

Usually in ants, there is a temporal division of labour: workers specialize with their age (Jeanne, 1986). However, the colony needs (*e.g.* more brood to feed, galleries to dig) can induce individuals to change caste – regardless of the age (Robinson, 1992). For instance, if most of the foragers die from predation, some of the nest-worker workers become foragers to maintain food supply of the colony. They become foragers earlier than expected. Therefore, we might find individuals of different age within the same caste. Age-related phenotype including physiological traits would consequently not be homogenous and thus explain part of the individual variability within a caste. However, we expect this age-independent caste switching effect to be minimal, since ants were reared under constant laboratory conditions for more than two years. Furthermore, all the workers used in this study were at least two years old, damping the potential impact on the proteome of a big age difference between worker castes. Although the effect of age is mitigated, we cannot be 100% sure that it does not influence our results, at least in part. Under this assumption, the age effect can be either independent or confounded with the effect of the caste. If it is independent, then it could explain the remaining variation not attributable to the caste (axis 2 of the first PCA and axis 1 of the second one). If age and cate effects are confounded, then this remaining variation would be due to a third factor, which remains unknown so far.

### 4.3. *A possibly multifactorial gap in lifespan*

#### 4.3.1. *Energy metabolism*

Proteins associated with energy metabolism were more abundant in worker ants. Most of the proteins forming this group are involved in the

Krebs Cycle (*e.g.* NADH dehydrogenase, succinyl ligase, citrate synthase), ATP synthesis (ATP synthase) or lipid beta-oxidation (long-chain-fatty-acids ligase 3). This suggests a higher metabolic activity (potentially associated with higher metabolic rates) in workers than in queen ants. Oxygen consumption at the colony level was found to be higher in workers than in queens of fire ants (Vogt and Appel, 1999). Oxygen consumption was even higher in workers moving the most intensively (Ferral et al., 2017) or the smaller ones (Calabi and Porter, 1989), raising the question whether metabolic rate is also an important determinant of lifespan in workers.

#### *4.3.2. Somatic maintenance*

Queen ants were, among others, characterised by higher amount of apoptosis-regulating proteins. As recently highlighted (van Deursen, 2014), preventing the accumulation of senescent cells within tissues is a key determinant of an organism's lifespan and health. Killing dysfunctional cells seems also to be one of the keys to longevity (Berger et al., 2006; Ravikumar et al., 2006; Tchkonia et al., 2013). The negative impact of senescent cells is mostly mediated through the Senescent Associated Secretory Pathway (Matjusaitis et al., 2016; Tchkonia et al., 2013). Contrarily, promoting senescence may also be beneficial through its implication in tissue repair (Jun and Lau, 2010) and tumour suppression (Collado et al., 2007). Focusing on the dynamics of senescence markers over life in the different castes and in different species may be of interest in the near future to estimate how senescence control has co-evolved with both longevity and sociality in ants. Queens also had higher amount of proteins belonging to the two functional groups 'Detoxification' and 'Chaperone'. In these groups are found proteins involved in macromolecules restoration after stress (*e.g.* aldehyde dehydrogenase, selenium-binding protein 1-a), ensuring proper folding of proteins (GrpE protein homolog, T-complex protein 1) or regulating cell energy production during stress (mitochondrial UCP2). Higher protein quantities from these groups characterised the queens. A previous study in *L. niger* has also found that the expression of somatic maintenance genes is up-regulated in queens (Graeff et al., 2007). This suggests that the queen's longevity might, at least in part, result from a higher energy investment in preventing cell damages.

#### *4.3.3. Reproduction and refinement of longevity trade-offs in queens*

As expected, the queen's reproductive role was confirmed by the analysis, since proteins related to reproduction were solely found in queen ants. The Reproduction functional group was only made of sperm-related proteins (*e.g.* sperm-associated antigen). This protein abundance can be explained by the spermathecal storage of sperm in queens. In our study and contrary to genomic studies (Graeff et al., 2007; Grozinger et al., 2007), vitellogenin was not overexpressed in the reproductive caste. As highlighted by Amdam et al. (2003), vitellogenin is also found in workers. The difference in protein quantity might not be sufficient to isolate workers from queens. During oogenesis, lipids are required for the biosynthesis of the egg cell membrane or lipoproteins (Engelmann, 1979), and a functional reproductive system synthesizes steroid hormones, which require lipid precursors (Hoffmann, 1980). Consistently, queens had on average a higher quantity of proteins in- volved in lipid transport (*e.g.* phospholipid-transporting ATPase, apo- lipoprotein D) or lipid synthesis (fatty acid synthase). Since energy is limited, investment in reproduction is done at the expense of other functions. Consequently, when a species or an individual is long-lived, we usually expect a lower energy investment in reproduction according to the fecundity/lifespan trade-off (Stearns, 1977). Queens of social insects do not seem to undergo this trade-off, as they are both long-lived and the only reproductive individual in the colony (Blacher et al., 2017). This is notably the case in black garden ant queens characterised by intense reproduction combined with extreme lifespan – up to 28 years in *L. niger* (Parker, 2010). The solution could be not to consider a lifespan-vs-fecundity trade-off, but a lifespan-fecundity-vs-immunity trade-off. An active immune system is energy-consuming both at the individual (Moret and Schmid-Hempel, 2000) and colony level (Evans et al., 2005). The investment in immunity has been shown to impair reproduction and/or growth (Kopp and Medzhitov, 2009). For instance, up-regulation of immune genes decreases reproductive success in urban blue tits (Capilla-Lasheras et al., 2017). Consequently, if the queen in- vests less in the immune system – as suggested by our data – she might save energy for reproduction or/and mechanisms aiming to avoid or repair damages to macromolecules.

#### *4.3.4. ToR pathway in social insects*

Proteins whose quantity differed between castes were downstream component and/or weakly related to the ToR pathway. We cannot therefore highlight a clear involvement of the ToR pathway in caste differentiation in black garden ants. Whereas, it is unequivocal in honey bee, where queen-destined larvae upregulate this signalling pathway relatively to worker-destined ones (Page and Amdam, 2007; Patel et al., 2007). As the queen-destined larvae are overfed, such a finding con- firms the nutrient-sensitive role of ToR pathway to control growth ac- cording to the food availability. On the other hand, activation of the ToR pathway is strongly associated with a shorter lifespan among diverse taxa (Kapahi et al., 2010). The longevity secret of queen social insects might be a ToR expression modulation depending on their age. When queens are still larvae, an active ToR pathway (stimulated by overfeeding) would allow somatic growth and ovaries maturation. Then, ToR expression would decrease with age, protecting the queens from senescence. The opposite scheme would take place in workers.

## 5. Conclusions

We showed that proteomics allows assessing fine molecular differ- ences induced by task specialization in a social insect species. The non- targeted screen of the whole proteome highlighted a wide diversity of caste-dependent functions from immunity to reproduction to digestion to insecticide resistance. Our study also raises evolutionary questions about longevity and energy trade-offs in eusocial species, beyond the classical free-radical theory of ageing. Thanks to our exploratory ap- proach, we now have a more global insight into all the functions that can be affected by the division of labour in a eusocial species. Some are well studied (*e.g.* social immunity), others less, especially in the adult stage (*e.g.* the ToR pathway, difference in the metabolism of digestion). We therefore hope to pave the way for future experiments to accurately test the numerous and diverse molecular mechanisms induced by a eusocial lifestyle.

### Author contributions

Conceptualization, M.Q., C.S., J-L.D., F.C., FB.; methodology, FB, J-LD, MQ.; software, M.B-D., F.B., M.Q.; validation, F.B., M.B-D., M.Q.; formal analysis, M.Q.; resources, J-L.D., F.B., C.S., F.C.; data curation, F.B., M.B-D.; writing—original draft preparation, M.Q., M.B-D., F.B.; writing—review and editing, C.S., J-L.D., F.C., F.B.; visualization, M.Q.; supervision, C.S., J-L.D., F.C., F.B.; project administration, C.S., J-L.D., F.C., F.B.; funding acquisition, M.B-D, F.C., F.B., C.S.

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**The authors declare no conflict of interest.**

## Appendix A. Supplementary data

The following charts and tables are available online. Fig. S1: PCAs used for variable selection (before clustering by biological function). Table S1: Raw data from the mass spectrometry-based proteome ana- lysis, Table S2: Proteins used for the PCAs with the biological functions amongst the three castes, Table S3: Proteins used for the PCAs with the biological functions amongst workers only, Table S4: Inertia's 95th percentile for the first two dimensions of 10,000 PCAs. Tables S1–S3 present raw data and are available in a separated online repository (https://doi.org/10.17632/xk4rpddxx6.1).
Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2019. 103907.

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# Chapter 5| A metabolomics study of task specialization in a social insect, the black garden ant (*Lasius niger*)

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The backside of every ant
Sparks -
Spring sunshine.

L'arrière-train de chaque fourmi
Étincelle -
Soleil de printemps.

Kaneko Tota

**Abstract**

Unlike other omics approaches (genomics, transcriptomics, proteomics), metabolomics does not focus on the study of a single family of molecules (nucleic acids, proteins) but on several at the same time (*e.g.* lipids, carbohydrates, vitamins, amino acids). Moreover, the turnover of metabolites is much higher than that of proteins or changes in gene expression. Metabolomics therefore has the potential to capture biological processes distinct from other omics techniques. Thanks to mass spectrometry, we were able to explore the influence of the division of labour in a social insect, the black garden ant (*Lasius niger*). Moreover, a former proteomics study done in the same species allowed us to compare the findings of both approaches. We found that queens, less exposed to pathogens and pollutants, had less metabolites related to immunity and seemed to undergo less oxidative stress. Thus, they would potentially extend their lifespan both by reducing oxidative damage but also by saving energy they can invest in other processes related to longevity or reproduction. On the other hand, foragers, confronted with the external environment, exhibited the exact opposite metabolomic profile. The nest-workers had larger amounts of metabolites related to nutrient absorption, suggesting that they may have a predominant predigesting role. Contrary to our predictions, we did not find any metabolite linked to reproduction in queens. This metabolomics analysis allowed us to highlight processes linking longevity and nutrient sensing that failed to be identified by proteomics. To our knowledge, this study is the first one to use metabolomics to understand the physiological consequences of division of labour, both between queens and workers, and between worker castes. It shows that thanks to the latest advances, metabolomics is now a powerful approach for conducting comparative functional studies, even in non-classical animal models. Nevertheless, we have also seen that databases still need to be completed to ease a more comprehensive view of the involvement of metabolites in biological processes.

**Keywords:** omics, mass spectrometry, division of labour**,** eusociality, lipid metabolism, nutrient sensing, comparative study

**1. Introduction**

Metabolomics is the science that analyses the metabolome, *i.e.* the set of final or intermediate products of metabolism, called metabolites: *e.g.* vitamins, lipids, nucleic acids, oligosaccharides, amino acids. Since the 2000s, metabolomics has considerably developed in insects, especially social insects (Snart *et al.* 2015), and has made it possible to refine our understanding of biological processes such as immunity (Aliferis *et al.* 2012), diapause and resistance to cold (Colinet *et al.* 2012), exposure to insecticides or pollutants (Derecka *et al.* 2013; Shi *et al.* 2018; Rothman *et al.* 2019), mutualism or parasitism interactions with bacteria and fungi (Wu *et al.* 2017; Birer *et al.* 2020; Li *et al.* 2020), and venom composition (Klupczynska *et al.* 2018). A large part of the literature using metabolomics in social insects deals with chemical communication through pheromones and cuticular hydrocarbons. Cuticular hydrocarbons serve as individual markers (Bonavita-Cougourdan *et al.* 1987; Liang and Silverman 2000; Wagner *et al.* 2000; Dani *et al.* 2001; Châline *et al.* 2005; Torres *et al.* 2007) and allow the recognition of colony, sex, age and caste (Singer 1998; Cuvillier-Hot *et al.* 2001; Greene and Gordon 2003). They also reflect the ovarian activity and, therefore, the reproductive status of individuals (Peeters *et al.* 1999; Dietemann *et al.* 2003; de Biseau *et al.* 2004). Pheromones and other glandular secretions have been intensively described and associated with a great variety of functions. For instance, several of them exhibit antibiotic properties (Beattie *et al.* 1986; Ortius-Lechner *et al.* 2000; Fernández-Marín *et al.* 2006; Tragust 2016), while others are territory or routes markers (Cammaerts *et al.* 1981; Jaffe and Puche 1984; Hölldobler *et al.* 2004), various alarm and defensive compounds have been identified (Regnier and Wilson 1968; Wheeler and Blum 1973; Hernández *et al.* 1999; Leclercq *et al.* 2000). These studies have led to a better understanding of the molecular language that governs the complex organisation of social insect colonies. However, there are still few comparative metabolomic studies in social insects actually addressing the interrelationship between the metabolome and the division of labour, except some studies focusing on the microbiome, which combine genomics and metabolomics (reviewed in Sinotte *et al.* 2020).

Depending on their social role, *i.e.* the caste they belong to, social insect individuals differ in their anatomy, behaviour, and physiology In most ant species, for example, the queen (or queens for polygynous species) is larger and the only one to be fertilised to ensure the sexual reproduction of the colony. The queen also differs from workers in her life span, on average ten times longer (Keller and Genoud 1997). Several studies have tried to understand the mechanisms underlying these differences in development and longevity. For example, a higher concentration of ecdysteroids and vitellogenin in reproductive individuals has been observed (Robinson *et al.* 1991; Gospocic *et al.* 2017), as well as higher expression levels for genes related to life extension and immunity (e.g. Graeff *et al.* 2007; Fang *et al.* 2012;

Begna *et al.* 2012; Lucas and Keller 2018). However, inter-individual differences are not restricted to queen versus workers, and also exist between worker castes. Workers perform a wide variety of tasks depending on the ecology of their species and specific morphological adaptations can even be observed (Morton Wheeler 1908; Jeanne 1986; Seeley 1986; Harvell 1994). Although some behaviours are species-specific, workers can be separated into two groups: on the one hand, the nest-workers that perform their tasks in the shelter of the nest (*e.g.* care of the queen and larvae, nest construction); and on the other hand, the foragers that bring food back from outside to the colony. It has been shown that belonging to the caste of the foragers accelerates the ageing rate (Chapuisat and Keller 2002; Kohlmeier *et al.* 2017) and, in bees, the transition from forager to nest-worker can restore immune and cognitive functions (Amdam *et al.* 2005; Baker *et al.* 2012; Münch and Amdam 2013). Besides, concentrations of vitellogenin, juvenile hormone and insulin/insulin-like growth factor not only reflect reproductive status but also task specialization within workers (Guidugli *et al.* 2005; Nelson *et al.* 2007; Azevedo *et al.* 2011; Corona *et al.* 2013; Libbrecht *et al.* 2013; Kohlmeier *et al.* 2018b). It is highly likely that these differences in anatomy, behaviour, longevity, and physiology alter the metabolism and thus the metabolome of individuals according to their caste (queens, nest-workers, foragers).

This study aimed to identify the metabolomic specificities of the division of labour among the castes of a social insect, the black garden ant (*Lasius niger*). Our comparison was not restricted to the usual opposition of queens vs. workers, but we focused also on the differences between worker castes, which have been little studied so far. The use of the black garden ant gave us a point of comparison with a previous proteomics study carried out under similar conditions in the same species (Quque *et al.* 2019). The cross-use of multi-omics data makes possible the capture of several aspects of the same phenomenon, *e.g.* different steps of regulatory mechanisms, and thus to have a better comprehension and a more detailed view (Gygi *et al.* 1999; Hunt *et al.* 2010; LeBoeuf *et al.* 2016). Based on this previous proteomic study and the literature cited above, we expected to find larger amounts of metabolites related to reproduction and somatic maintenance in queens. Regarding the immune system, genomic studies generally show overexpression in queens, however, our previous proteomic study did not support this conclusion. It was therefore interesting to evaluate whether metabolomics is more in line with genomics or proteomics in such context. Concerning the differences between workers, based on the previous proteomics study in *L. niger*, we predicted the foragers to have more metabolites related to xenobiotic detoxification and nest-workers to have more metabolites related to food absorption and digestion.

## 2. Methods

### *2.1. Ant keeping and preparation of samples before metabolomics*

The black garden ant (*Lasius niger*, Linnaeus 1758) is a common species in Western Europe and is found widely in urban areas (Konorov *et al.* 2017). There is no dimorphism between the worker castes, with individuals of about 3 mm long-living for up to 3 years. Queens can reach 7-9 mm and live on average 20 years (Hölldobler and Wilson 1990). Adult colonies are monogynous (only one queen lays eggs). For our study, wild newly mated queen ants were collected in Strasbourg, France (N 48.5893, E 7.7512) in July 2018 and then placed individually in glass tubes in the dark. Nineteen queens established a viable colony. Colonies were kept at a temperature of 21°C at night and 26°C during the day, relative humidity of 50-60%, and the photoperiod mimicked the natural photoperiod of the capture area. The ants were fed with a 0.3 M sugar water solution and mealworms.

Since we were interested in the differences between foragers and nest-workers and there is no dimorphism between them, we separated them according to their behaviour. To do this, food sources were removed for 48 hours, then 1 M sugar water was supplied. Once the first forager discovered the food source, we waited five minutes for optimal recruitment of the largest number of foragers. After these five minutes, all the ants that came to the food source were collected and marked on the abdomen with an acrylic ink (Posca ©). This protocol was carried out three times to make sure that all the foragers were recruited, but with a four-day interval to allow the colony to rest.

Because of the small amount of biological material represented by one ant, one sample analysed by mass spectrometry consisted of a pool of several ants. The queens were pooled in pairs and the workers (foragers and nest-workers) in groups of 50. In order to prevent a possible bias owed to different origins, the pools contained a balanced mixture of the 19 colonies. In total, 8 samples from each caste were formed, *i.e.* using 16 queens, 400 foragers and 400 nest-workers. Prior to mass spectrometry analysis, the ants were snap-frozen in liquid nitrogen. The ink on foragers' abdomens was removed with acetone. The ants were ground under liquid nitrogen for 1 min at 30 Hz with steel beads (Mixer Mill MM400, Retsch, Eragny Sur Oise, France). Tubes containing the resulting powder were stored at -80°C until analysis.

### *2.2. Metabolomic analysis*

Deionised water was filtered through a Direct-Q UV (Millipore) station, isopropanol and methanol were purchased from Fisher Chemicals (Optima ® LC/MS grade). Deuterium labelled [$^{2}H_{6}$](+)-cis,trans-

abscisic acid ($^2H_6$-ABA) (OlChemIm). NaOH was obtained from Agilent Technologies, acetic acid formic acid from Sigma Aldrich.

2.2.1. Sample preparation

30 mg of ground ants powder from each caste was resuspended in 1ml cold (5°C) methanol spiked with an internal standard of deuterium labelled [$^2H_6$](+)-cis,trans-abscisic acid ($^2H_6$-ABA) at 0.1µg/ml. After 10 seconds of vortexing, the samples were stored for 16h at -20°C, then centrifuged at 13000 rpm for 15 min at 4°C. The supernatant was collected and dried by sublimation using a SpeedVac concentrator (Savant SPD121P, Thermo Fisher). The samples were resuspended in 200µl of methanol and analysed in liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) described in section 2.2.2.

2.2.2. LC-HRMS analysis

Samples were analysed using liquid chromatography coupled to high-resolution mass spectrometry on an UltiMate 3000 system (Thermo) coupled to an Impact II (Bruker) quadrupole time-of-flight (Q-TOF) spectrometer. Chromatographic separation was performed on an Acquity UPLC ® BEH C18 column (2.1x100mm, 1.7µm, Waters) equipped with an Acquity UPLC ® BEH C18 pre-column (2.1x5mm, 1.7µm, Waters) using a gradient of solvents A (Water, 0.1% formic acid) and B (MeOH, 0.1% formic acid). Chromatography was carried out at 35°C with a flux of 0.3mL.min$^{-1}$, starting with 5% B for 2 minutes, reaching 100% B at 10 minutes, holding 100% for 3 minutes and coming back to the initial condition of 5% B in 2 minutes, for a total run time of 15 minutes. Samples were kept at 4°C, 10µL were injected in full loop mode with a washing step after sample injection with 150µL of wash solution ($H_2O$/MeOH, 90/10, v/v). The spectrometer was operated in positive ion mode on a mass range of 20 to 1000 Da with a spectra rate of 2Hz in AutoMS/MS scan mode. The end plate offset was set at 500 V, capillary voltage at 2500 V, nebulizer at 2 Bar, dry gas at 8 L.min$^{-1}$ and dry temperature at 200°C. The transfer time was set at 20-70µs and MS/MS collision energy at 80-120% with timing of 50-50% for both parameters. The MS/MS cycle time was set to 3 seconds, absolute threshold to 816 cts and active exclusion was used with an exclusion threshold at 3 spectra, release after 1 min and precursor ion was reconsidered if the ratio current intensity/previous intensity was higher than 5. A calibration segment was included at the beginning of the runs allowing the injection of a calibration solution from 0.05 to 0.25min. The calibration solution used was a fresh mix of 50mL isopropanol/water (50/50, v/v), 500µL NaOH 1M, 75µL acetic acid and 25µL formic acid. The spectrometer was calibrated in high precision calibration (HPC) mode with a standard deviation below 1ppm before the injections for each polarity mode, and re-calibration of each raw data was performed after injection using the calibration segment.

2.2.3. Metabolite annotation and quantification

Raw data were processed in MetaboScape 4.0 software (Bruker): molecular features were considered and grouped into buckets containing one or several adducts and isotopes from the detected ions with their retention time and MS/MS information when available. The parameters used for bucketing were a minimum intensity threshold of 10000, a minimum peak length of 4 spectra, a signal-to-noise ratio (S/N) of 3 and a correlation coefficient threshold set at 0.8. The $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ ions were authorised as possible primary ions. Replicate samples were grouped and only the buckets found in 80% of the samples of one group were extracted from the raw data. The obtained list of buckets was annotated using SmartFormula to generate raw formula based on the exact mass of the primary ions and the isotopic pattern. The maximum allowed variation on the mass ($\Delta m/z$) was set to 3ppm, and the maximum mSigma value (assessing the good fitting of isotopic patterns) was set to 30. To put a name on the obtained formulae (*i.e.* annotate), analyte lists were derived from FooDB (http://foodb.ca), LipidMaps (https://www.lipidmaps.org) and SwissLipids (https://www.swisslipids.org/). The parameters used for the annotation with the analyte lists were the same as for SmartFormula annotation.

*2.3. Metabolite functional and structural classification*

To interpret metabolomics data, we looked for the biological processes in which the differentially expressed metabolites were involved, and we classified them according to chemical class (*e.g.* sphingolipids, amino acids, fatty acids). We proceeded in two steps. First automatically, by retrieving data either from Kyoto Encyclopedia of Genes and Genomes database (KEGG; biological processes, https://www.genome.jp/kegg) or from the use of ChemRICH (metabolite classes, Barupal and Fiehn 2017), then manually when the automatic method did not work. For biological processes, the manual completion method consisted of searching the literature for articles relating to the metabolite concerned or metabolites of the same type (references indicated in tables). To complete the classes of metabolites not found automatically, we used the sub-class of the "Chemical Taxonomy" section of the HMDB database (https://hmdb.ca). If the metabolite was not present in HMDB, the subclass from the "Classification" section of the FooDB database (https://foodb.ca) was used.

*2.4. Data filtering and statistics*

A metabolite was considered present for sure in a given caste only when it was present in at least 80% of the samples from this caste, *i.e.* 6 samples out of 8. Conversely, a metabolite was considered completely absent from a given caste only when none of the samples from this caste contained the metabolite. Hence, all metabolites found in 1 to 5 out of 8 samples were excluded from the analyses. In the resulting dataset, the missing data (metabolites present in 6 or 7 out of 8 samples) were imputed using an iterative PCA (principal component analysis) algorithm (MissMDA package v.1.17, Josse and

Husson 2016). Missing data represented 2.6% of the data analysed. When all three castes are considered, there is a greater probability that a metabolite is present in less than 80% of the samples for at least one of the three castes. Therefore, when comparing worker castes with each other, we did not consider the queen samples to filter the related dataset. This resulted in a significant increase in the number of metabolites (see results section) for the comparison between nest-workers and foragers, and thus gave further information about the underlying metabolomic differences.

In a first step, we aimed at exploring the different sources of variation among our samples, both inter- and intra-caste. For this purpose, we performed a PCA with the FactoMineR package (v.2.3, Lê *et al.* 2008). The data were standardised (centred and scale) before running the PCA. Only the metabolites correlated more than 50% to the axes and with a $cos^2$ greater than 0.8 were retained. The results of this preliminary PCA are available in electronic supplementary materials: table with metabolite identification, classification, and correlation to the axes (**ESM1**, Table S5), as well as the PCA plot (**ESM2**, Figure S1). Those most informative and discriminating metabolites were then grouped according to chemical class. We ran a second PCA with the chemical class as explanatory variables to highlight the typical metabolites for each caste of the black garden ant. Here, we integrated the absent metabolites because their null values were averaged with the other metabolites of the same class.

The PCA provided a global picture of the inter- and intra-caste variation observed in the three castes studied, based on chemical classes of metabolites. We wanted to refine this global picture by highlighting the metabolites that differed most strongly between the castes compared two-by-two. For this purpose, we calculated $log_2$ fold-changes (further referred to as Log2FC) for each metabolite using the DESeq2 package (v.1.28, Love 2014). In this analysis, we retained only the metabolites with a false discovery rate (FDR) lower than 0.05 and a Log2FC higher than 2 (up-regulated) or lower than -2 (down-regulated). Absent metabolites could not be used here because of the incompatibility of a null value with the log function. We built heat maps with the ComplexHeatmap package (v.2.42, Gu *et al.* 2016). In order to better highlight the metabolic pathways involved, we used the online tool MetaMapp (Barupal *et al.* 2012). This online software builds the biochemical relationships between all identified metabolites by combining the information available in the following databases: KEGG reaction, Tanimoto chemical, and NIST mass spectral similarity scores. Then, it provides matrices readable by the free software Cytoscape (https://cytoscape.org/) which allows the construction of network graphs associated with the identified metabolites. We presented in the section "results" only the networks showing at least one metabolite present in a significantly (FDR < 0.05) and strongly (Log2FC > 2 or < -2) different quantity in the targeted caste compared to the control one. Unless otherwise specified, the analysis and graphical representations were made using R software, version 4.0 (R Core Team 2019).

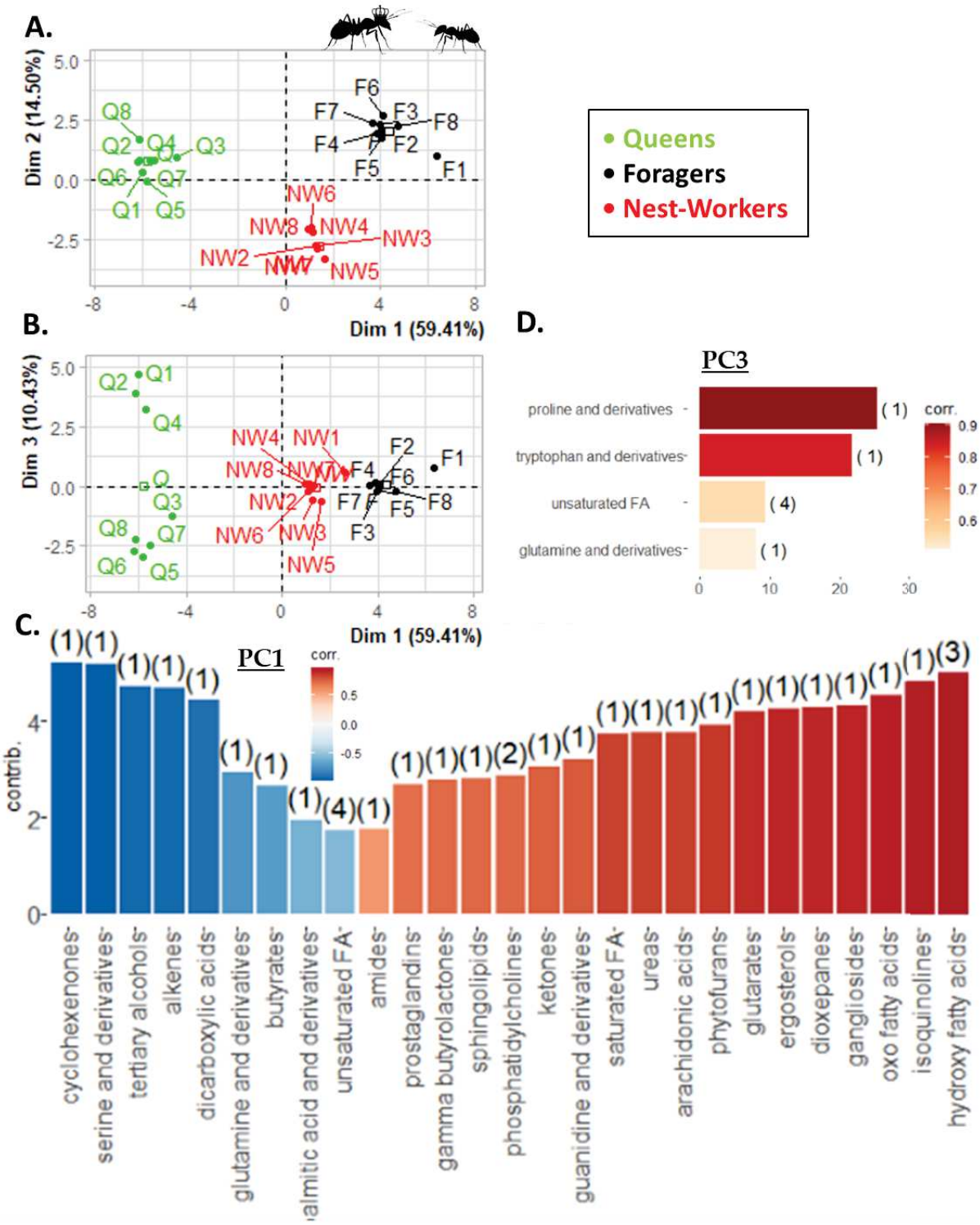


**Figure 1. Classification-based PCA amongst the three castes of *L. niger* (121 metabolites).** We kept the metabolites from the preliminary PCA (**Fig. S1 Left**), grouped them according to their class and ran a PCA with the classes as variables. **A:** PC1 and PC2. **B:** PC1 and PC3. The bar plots represent the classes of metabolites and their correlation with PCs from blue (negatively correlated) to red (positively correlated), as well as their contribution (bar length) and the number of metabolites in a given class (in brackets). Information is given for each principal component: PC1 (C) and PC3 (D). All metabolites showed are correlated more than 50%. The list of metabolites is available in Table S6 (ESM1). FA stands for fatty acids.

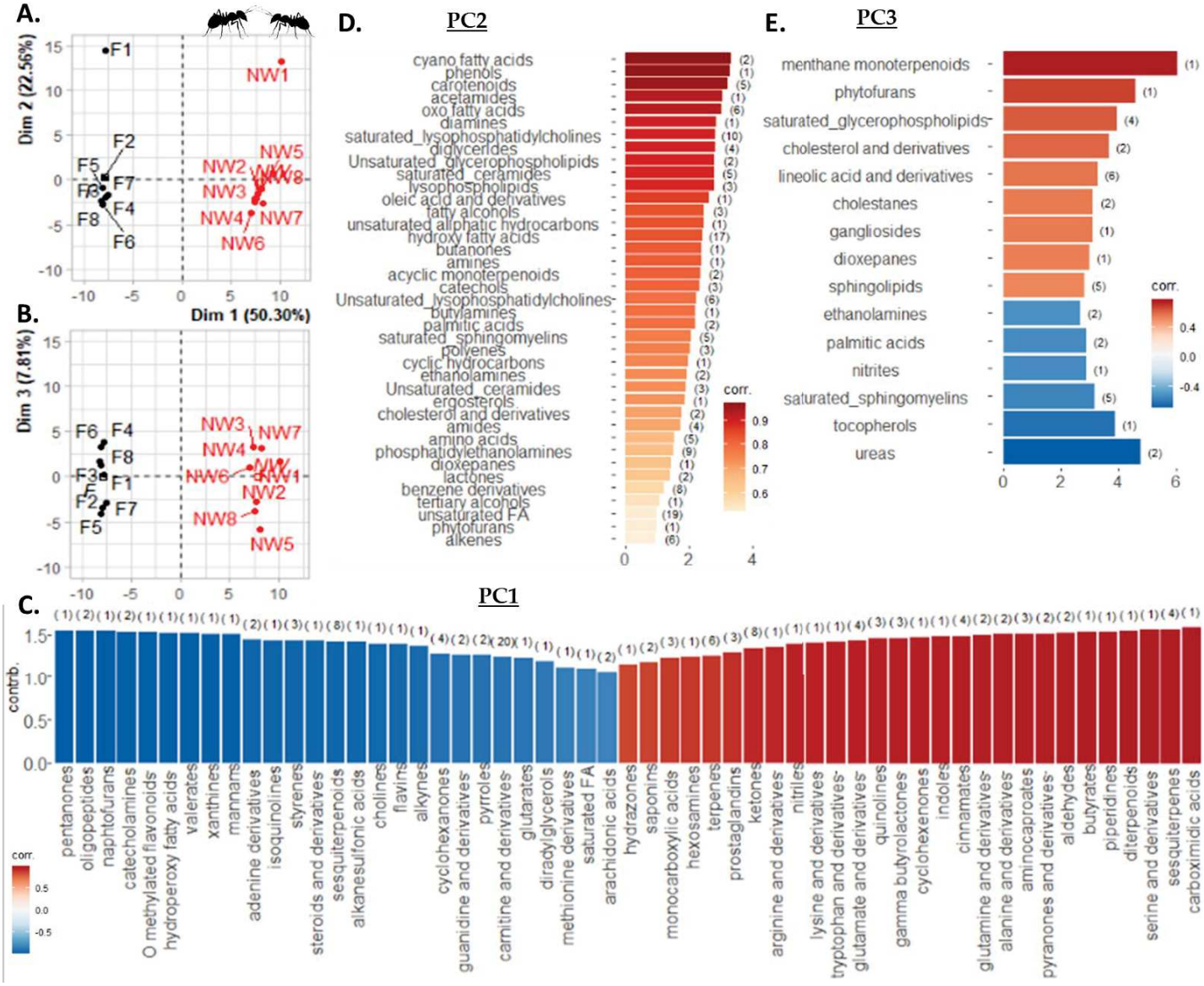


**Figure 2. Classification-based PCA within the worker castes (1807 metabolites).** We kept the metabolites from the preliminary PCA (Fig. S1 Right), grouped them according to their class and ran a PCA with the classes as variables. **A:** PC1 and PC2 within the worker castes only. **B:** PC1 and PC3 within the worker castes only. The bar plots represent the classes of metabolites and their correlation with PCs from blue (negatively correlated) to red (positively correlated), as well as their contribution (bar length) and the number of metabolites in a given class (in brackets). Information is given for each principal component: PC1 (**C**), PC2 (**D**) and PC3 (**E**). All metabolites showed are correlated more than 50%, except for PC1, with which the correlation is at least of 80%. The list of metabolites is available in Table S6 (ESM1).

## 3. Results

The mass spectrometry analysis (LC-MS/MS) revealed 1991 metabolites in the three studied castes of the *Lasius niger* ant, *i.e.* queens, foraging workers, and nest workers. When considering the three castes, 121 metabolites were present in at least 6/8 samples for each caste, and 97 metabolites were completely absent from at least one caste. When considering only the workers, 1807 metabolites were present in at least 6/8 samples for each caste, whereas 52 were completely absent from foragers or nest-workers. Out of the 1991 identified metabolites, 486 were automatically annotated. These annotations mainly came from FoodDB and LipidMAPS metabolomics databases. Original data sets are available in the electronic supplementary material (**ESM1,** Table S1-S3). The references for the biological processes to which we refer below can be found in ESM1 (**Table S4-S7**). For a better understanding of the results below, we draw the reader's attention to two points of terminology. The first concerns the names of the ant castes. We have studied three of them: the queens, foragers and nest-workers. When the term 'workers' is used alone, it refers to both nest-workers and foragers, as opposed to queens. The second point concerns the naming of metabolites. In metabolomics, the definitive identification of a given molecule is ensured by comparison with a reference standard. However, we did not use a standard for the 1991 metabolites in this study. The simple comparison of the signal obtained with an online database is called annotation: level 3 of Schymanski's classification (Schymanski *et al.* 2014). Consequently, when a metabolite is referred to as annotated, it means named and not functionally annotated, contrary to the terminology used in other fields.

### *3.1. Absent metabolites*

Table S4 (**ESM1)** lists all the annotated metabolites absent from at least one caste both for the analysis that considered the three castes (**Table S4 A**) and the analysis of workers only (**Table S4 B**) comparisons. Queens, when compared to workers, lack metabolites related to oxidative damage, immune system, mandibular secretion, and nutrient availability signalling. Foragers are the only ones, when compared to the two other castes, to have 2S-amino-3R,4R,5S-trihydroxy-2-(hydroxymethyl)-14-oxo-eicos-6E-enoic acid (a sphingolipid) and the F-2-alpha prostaglandin. When comparing the two worker castes solely, only the foragers had metabolites completely absent from nest-workers. Among these metabolites, some were related to oxidative damages (methyl 5-hydroperoxy-6,8,9,11-bisepidioxy-12,14-eicosadienoate) and associated buffering pathways (1-nonadecene-2,3R-dicarboxylic acid, Prostaglandin F-2-alpha), others were terpenoids and sphingolipids, involved in numerous metabolic pathways (see **Table S4 B**). The 4-Ethyl-7,11-dimethyldodeca-trans-2-trans-6-1-o-trien-1-al might be a defensive secretion against predators.

*3.2. Inter- and intra-caste variation assessed through a classification-based PCA*

When considering the three castes of black garden ants, the first, second and third principal components (PC1, PC2 and PC3) respectively explained 59.1 %, 14.5 % and 10.4 % of the total variation (84.07 % of total variance). Workers, and especially foragers, were positively correlated with PC1, while queens were negatively correlated with it (**Figure 1 A**). Hydroxy fatty acids, isoquinolines, oxo fatty acids, gangliosides, dioxepanes, ergosterols derivatives, glutarates, phytofurans, arachidonic acid derivatives, ureas, saturated fatty acids, guanidine and derivatives, ketones, phosphatidylcholines, sphingolipids, gamma butyrolactones, prostaglandins were found in larger amounts in workers especially foragers (positively correlated to PC1). On the contrary, glutamine and derivatives, dicarboxylic acids, alkenes, tertiary alcohols, serine and derivatives, and cyclohexenones were negatively correlated to PC1 and thus typical of queens (**Figure 1 C**). The inter-queen variations appeared to depend mainly on metabolite classes along PC3: proline and tryptophan derivatives (**Figure 1 B and D**). We do not discuss PC2 because it separated the worker castes and this difference is studied more precisely in the dedicated PCA below. However, the complete list of all metabolites, including PC2 of this PCA is available in Table S6 (**ESM1**).

When considering the metabolic differences among the workers only, PC1, PC2 and PC3 respectively explained 50.3 %, 22.6 % and 7.8% of the variation (80.7% of total variance). The list of metabolites correlated to the different axes being too long to be quoted, we invite the reader to see **Figure 2 C, D** and **E**, as well as the online supplementary Table S6 (**ESM1)**. While PC1 clearly segregated the foragers (negative values) from the nest-workers (positive values), PC2 and PC3 highlighted inter-worker metabolic differences, with F1 and NW1 being markedly distinguished from the other worker samples (**Figure 2 A and B**).

*3.3. Pairwise comparison of ant castes' metabolomes*

For each pairwise comparison (foragers *vs.* queens, foragers *vs.* nest-workers and nest-workers *vs.* queens), Table S7 (**ESM1**) identifies the metabolites significantly (FDR < 0.05) and strongly down-regulated (log2FC < -2) or up-regulated (log2FC >2) and provides the related molecular formula, chemical class, and biological processes.

Like the PCAs, the heat maps (**Figures 4 and 5**) constructed from the 50 most expressed metabolites confirm that the metabolic differences allow the three castes tested in the black garden ant to be clearly separated by this blind hierarchical classification. There were, however, intra-caste variations similar to those highlighted by the PCA, *e.g.* the queen sample Q3 was separated from the other queen samples).

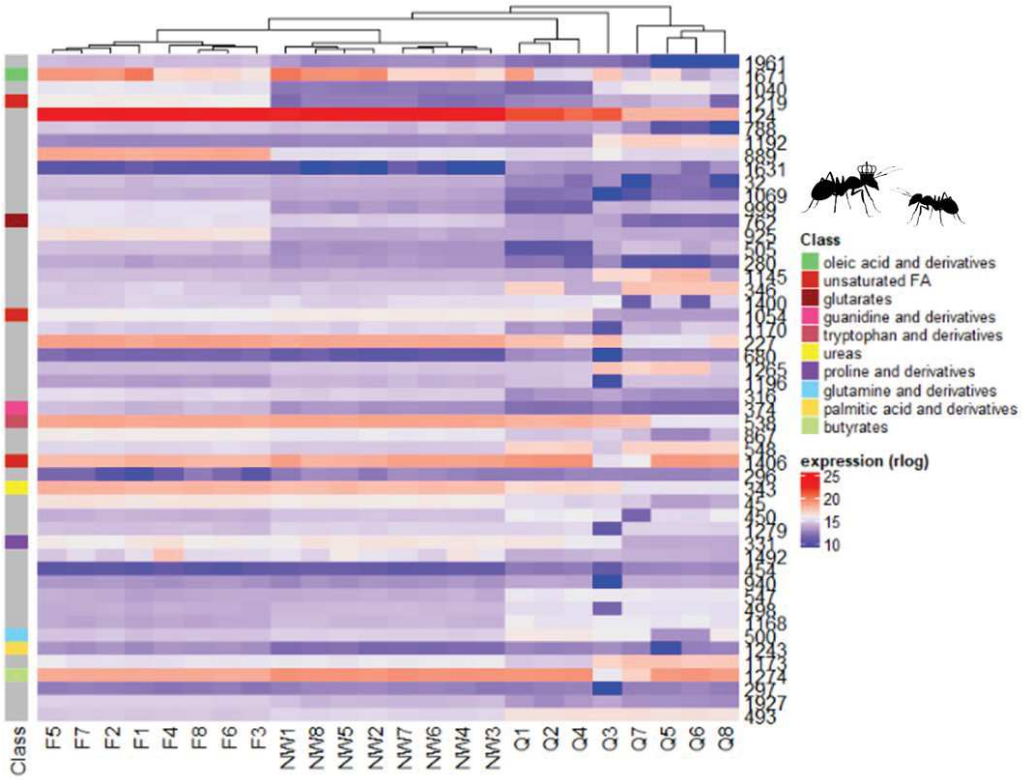


**Figure 4. Heat map of the 50 most expressed metabolites amongst the three castes of *L. niger*.** The left column indicates the metabolite class. Grey is a NA value. At the bottom are the sample number (F: forager, NW = nest-worker, Q = queen). The right column indicates the ID rather than the full name for legibility reasons (correspondence is indicated in every table provided). All metabolites here have a FDR < 0.05 and |Log2FC| > 2.

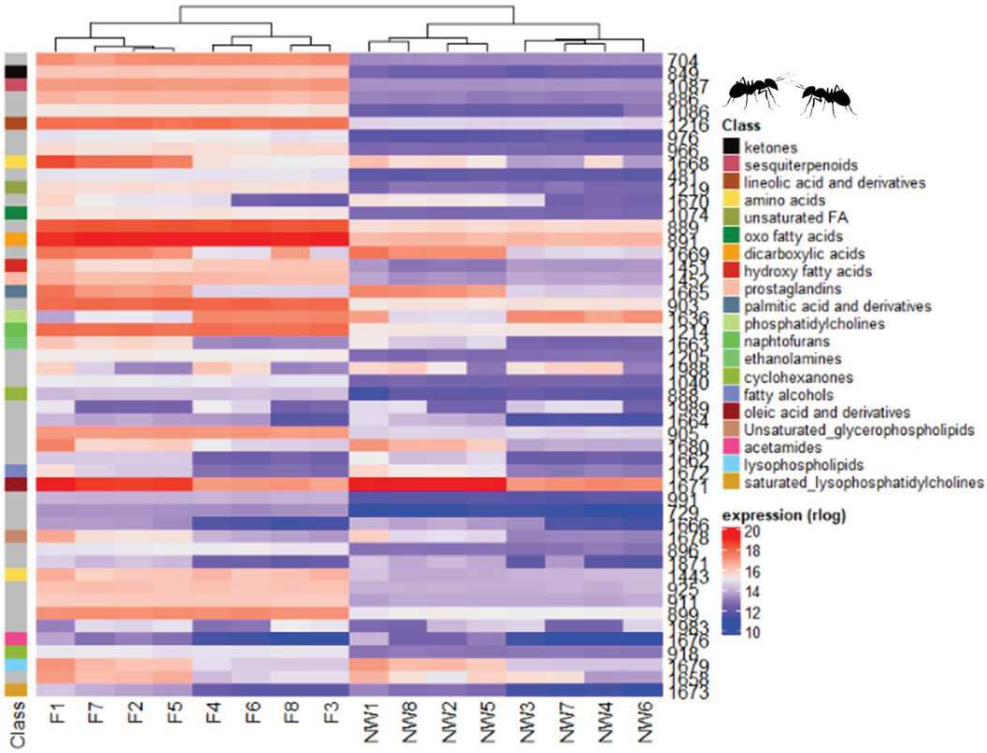


**Figure 5. Heat map of the 50 most expressed metabolites amongst workers only in *L. niger*.** The left column indicates the metabolite class. Grey is a NA value. At the bottom are the sample number (F: forager and NW = nest-worker). The right column indicates the ID rather than the full name for legibility reasons (correspondence is indicated in every table provided). All metabolites presented here have a FDR < 0.05 and |Log2FC| > 2.

Regarding the metabolic differences in workers, the metabolites less abundant in foragers compared to nest-workers mainly belonged to the classes of phosphatidylcholines, prostaglandins, carnitine and derivatives, and alkenes. There were metabolites with antifungal activity (pipericine), or linked to membrane and lipid transport : *e.g.* (3-[(4Z)-dec-4-enoyloxy]-4-(trimethylazaniumyl) butanoate, 2,3,4,5 - tetranor-9S,11R,15S-trihydroxy-13E-prostenoic acid). The metabolites more abundant in foragers compared to nest-workers belonged to several kinds of fatty acids, naphtofurans dicarboxylic acids, cyclohexanones, amino acids, prostaglandins, sesquiterpenoids, arachidonic acid derivatives, pentanones, and ketones. Those metabolites are known to be mostly embedded in the cell membrane and involved in cell signalling, notably through inhibition of protein kinase C.

When comparing foragers to queens, the following classes of metabolites were found up-regulated: glutarates, guanidine and derivatives, oleic acids, unsaturated fatty acids, ureas, and phosphatidylcholines. It was worth noting the presence of isobutylidene, a compound of pesticides or soil fertilisers. We also found a worker Dufour's gland secretion, the 9Z-octadecenamide. Other metabolites found are known to be embedded in the cell membrane or involved in amino acid metabolism (valine, leucine, isoleucine, arginine, proline).

We found few metabolites in greater abundance in nest-workers than in queens. They belonged to metabolite classes already highlighted in the comparison of foragers *vs.* queens: glutarates, oleic acid and derivatives, and ureas. The only annotated metabolite found up-regulated in nest-workers was also up-regulated in foragers *vs.* queens: the isobutylidene.

No metabolite was significantly found in lower quantity in workers compared to queens.

**4. Discussion**

Our metabolomic analysis combining principal component analysis (PCA) and $\log_2$ fold change calculation (Log2FC) highlighted distinct metabolomic profiles between the castes of the black garden ant (*Lasius niger*). In addition to inter-castes differences, we also underlined intra-caste variation in the metabolomes of queens, foragers, and nest-workers. The exhaustive lists of metabolites are available in Electronic Supplementary Material (**ESM1**). In the discussion below, we first stress metabolites involved in functions or life-history traits underlining the physiological and behavioural specificities of each caste. Then, we address the more general question of hormone and pheromone synthesis. Finally, we discuss the intra-caste variation and the metabolites related to reproduction in queens in the context of the low proportion of metabolites that were annotated and associated with a biological process. All along with the discussion, we compare the present metabolomics results with data from a previous proteomics study in *L. niger,* sharing the same experimental design (Quque *et al.* 2019).

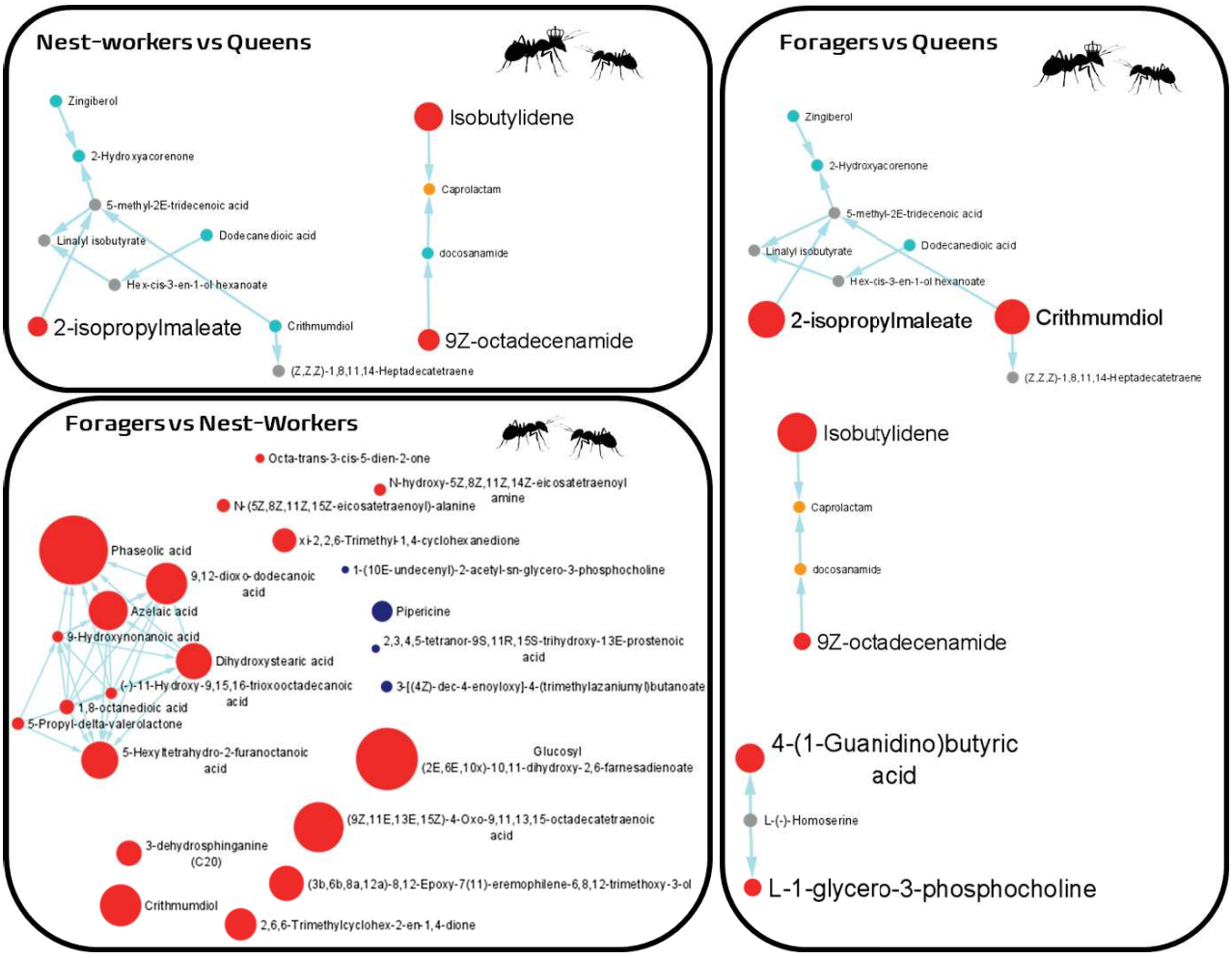


**Figure 6. Metabolomic networks for pairwise comparison of castes.** The caste compared is always the first to appear in the title and the reference caste, the second one. For example, isobutylidene is found in larger amount in nest-workers than in queens. We mapped the identified metabolites according to biochemical reactions with MetaMapp and visualised the resulting networks through Cytoscape. An arrow indicates a chemical transformation into the metabolite at the end of the arrow, based on known enzymatic reactions (KEGG reactant pair database) or on chemical similarity (Tanimoto chemical and NIST mass spectral similarity scores). The networks without significative and strong metabolomic difference between castes are not shown. As the comparison between worker castes involved too many metabolites (360) for a suitable display, we show only the metabolites with FDR < 0.05 and Log2FC > 2 or < -2, and not all the interactions in which they are involved. Dark blue (dark red) indicates a smaller (larger) quantity of a given metabolite with a Log2FC < -2 (Log2FC >2). Light blue (orange) codes for a decreased (increased) quantity of metabolite but with -2 < Log2FC < 0 (0 < Log2FC < 2). Grey indicates an absence of significative change (FDR > 0.05). The larger the dot size, the higher the absolute value of Log2FC is (min = -3.09, max = 6.64).

### *4.1. Queens are protected from stress, pathogens and pollutants*

Log2FC showed that isobutylidene, a soil fertiliser and insecticide compound (Hughes 1976; Retnakaran and Wright 1987), was present in larger amounts in workers than in queens. This reflects the capacity of workers to serve as a buffer against detrimental incomes from outside the anthill. The interactions among colony members probably prevent the queen from being in contact with high doses of noxious xenobiotics. Such a benefit to the queen has been described under the name of *social immunity*

in the context of pathogenic infections (Cremer *et al.* 2007; Walker and Hughes 2009; Hamilton *et al.* 2011; Le Conte *et al.* 2011; Aanen 2018). If queens are less exposed to outside threats, we could therefore understand that they invest less in the immune system and that we found some missing metabolites in queens potentially related to immunity. Indeed, the classification-based PCA revealed that arachidonic acid derivatives were found in larger amounts in workers than queens. For example, N-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoyl amine was completely absent from queens but present in both foragers and nest-workers. Arachidonic acid and derivatives are major components of the insect cuticle and are thought to be involved in the formation of nodules during melanisation (Stanley-Samuelson *et al.* 1991; Miller *et al.* 1994), the main insect immune response to bacterial infections (Tapia *et al.* 2014; Nakhleh *et al.* 2017). Moreover, 3-Ethyl-5-methyl-1,2-cyclopentanedione was also lacking in queens. This ketone is produced by bacteria and has antifungal and antibacterial activity (Abdel-Shafi 2017). The presence of communities of bacteria or fungi on ant cuticle has already been described and contributes to their immune defences (Feldhaar and Gross 2008; Konrad *et al.* 2015), as well as the caste recognition, reflecting the division of labour (Zientz *et al.* 2005; de Souza *et al.* 2013; Sinotte *et al.* 2020). Here metabolomics brings supporting pieces of evidence to our previous proteomic study (Quque *et al.* 2019) where we found the queens to be characterised by lower amounts of proteins linked to the immune system (*e.g.* T-cell immunomodulatory protein, ferritin) when compared to workers. We hypothesise that the immune protection granted by social immunity to social insect queens allows them to reduce the energetic investment in their own immune system, likely for the benefit of reproduction and somatic maintenance. At the inter-specific level, phylogenetic studies support the idea that the social structure may incur a reduced immune system with the evolution of eusociality: a larger colony size is associated with a weaker melanisation response (López-Uribe *et al.* 2016), social insects have fewer genes involved in immune functions than less social insects (Libbrecht *et al.* 2013), and a decrease in immune gene activity and diversity during the evolution of termites has also been evidenced (He *et al.* 2020). On the other hand, at the intra-specific level, the results in social insects are equivocal, finding up-regulation of gene expressions involved in immunity in workers (Judice *et al.* 2006; Lucas *et al.* 2017b) or queens (Graeff *et al.* 2007; Grozinger *et al.* 2007), or both depending on the worker caste compared (Stucki *et al.* 2017). As our results are obtained through proteomics and metabolomics, the divergent conclusions might exemplify how gene expression can be modulated downstream. This hypothesis would need an experimental design with concomitant analyses of the transcriptome, proteome, and metabolome to be properly tested. Genes, proteins, and metabolites may be involved in several processes. For example, as stated by Graeff and collaborators (2007), the up-regulation of a histone 2A homologue in *L. niger* queens may be related to immunity but also to a high rate of cell division. Vitellogenin is strongly associated to caste differentiation in social insects (e.g. Guidugli *et al.* 2005; Nelson *et al.* 2007; Kohlmeier *et al.* 2018b)

and the up-regulation in queens found in some studies (e.g. Graeff *et al.* 2007; Grozinger *et al.* 2007) might be more explained by the reproductive status than by the selection of the immune properties. Supporting this hypothesis, male honey bees (drones) have been show to maintain an effective immune response at different life stages even in the absence of vitellogenin synthesis (Gätschenberger *et al.* 2012). Similarly in our study, arachidonic acid derivatives are involved in many processes (e.g. cell membrane components, cellular signalling, pheromone synthesis; Blomquist *et al.* 1991) and their up-regulation in workers (more specially the foragers) might not be linked to the immune system. In future studies, directly challenging the immune system of queens and workers with controlled pathogens would provide more definite answers.

Out of six metabolites completely absent from queens, two of them were known to be related to oxidative damages. Xi-salsolinol has been shown to be a neurotoxin (Mravec 2006; Quintanilla *et al.* 2016) and to cause oxidative damage to cytochrome C (Kang 2013). (E)-12-(5-ethyl-4-hydroxytetrahydrofuran-2-yl)-9,12-dihydroxydodec-10-enoic acid belongs to the class of phytofurans that are known biomarkers of peroxidation of polyunsaturated fatty acids (PUFA) in plants (Cuyamendous *et al.* 2015, 2016; Yonny *et al.* 2016). As we have seen above, the social structure of the colony means that the queens are less exposed to pollutants and pathogens which are two sources of oxidative stress (Eeva *et al.* 2000; Smith *et al.* 2000; Torres and Velando 2007; Tkachenko and Kurhaluk 2012; Marri and Richner 2015). This could explain why the queens show fewer metabolites associated with oxidative damage. Moreover, less oxidative stress in queens would require a less active antioxidant system. This assumption agrees with the lower enzymatic antioxidant activity previously found in *L. niger* (Parker *et al.* 2004).

Finally, N-(5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl)-ethanolamine, a.k.a. EPEA, is another metabolite we did not detect in queens. EPEA has been found with an omega 3 fatty acid (DHA) to induce apoptosis and autophagy through PPARγ activation in cancer cells (Rovito *et al.* 2013, 2015). According to the longer lifespan of queens and the positive association between autophagy and longevity described in diverse taxa (Cuervo *et al.* 2005; Bergamini *et al.* 2007; Morselli *et al.* 2010; Guo *et al.* 2018), we would not expect this metabolite to be absent from queens. Yet, EPEA has also been shown to decrease thermal resistance and lifespan in *C. elegans* (Elphick 2012), where it probably acts as a sensor of nutrient availability and energy state (Connor and Watts 2019). Together these studies appear to point out nutrient-sensitive but autophagy-independent anti-ageing mechanisms in ant queens.

*4.2. Among workers, nest-workers up-regulate pathways linked to nutrition and longevity*

Compared to foragers, protein biosynthesis appeared to be more active in nest-workers, since the classification-based PCA found several amino acids and derivatives to be up-regulated, including

serine, alanine, glutamine, glutamate, tryptophan, lysine, arginine, and proline. Only methionine and its derivatives were found in larger amounts in foragers. Serine, glutamate, tryptophan, tyrosine, alanine and derivatives appeared to share common metabolic maps, all related to digestion and absorption of nutrients. In fact, they belong to one or more of the following KEGG maps: protein digestion and absorption (map04974), bile secretion:digestive system (map04976) vitamin digestion and absorption (map04977), mineral absorption (map04978). Similarly, Log2FC showed larger amounts of piperidine and carnitine in nest-workers, both metabolites notably involved in nutrient absorption (map04974 and map04976). These findings echoed those from our previous proteomic study (Quque *et al.* 2019), in which nest-workers overexpressed proteins linked to digestion (*e.g.* alpha amylase) when compared to foragers. As far as we know, the importance of digestion in ant nest-workers had not yet been reported and should be further investigated to specify the meaning and depth of greater amounts of digestion-related metabolites and proteins. We may assume that the storage of food excess by nest-workers (Lenoir 1981) followed by pre-digestion, would make it quickly available for further use, thus enhancing the fitness of the whole colony by buffering environmental unpredictability in food resources, but also by increasing food processing by conspecifics. Nest-workers may also pre-digest food for castes that do not perform this task very well. According to several studies, ant larvae do not require help to digest food (Went *et al.* 1972; Cassill *et al.* 2005; Erthal *et al.* 2007). The pre-digested food could be more valuable to queens, allowing them to invest less in digestive metabolism and save energy for other costly life-history traits such as reproduction and a long life span.

Among workers, nest-workers also exhibited up-regulated glutamic acid and nicotinamide. Glutamic acid is involved in numerous pathways, notably the metabolic pathways of other amino acids (see KEGG maps in **ESM1**, *e.g.* Table S7), but it is worth noting also its implication in nutrient sensing and metabolic activity through the activation of the FOXO transcription factor pathway (map04068). This activation is mediated by MK-801 glutamate receptor that dephosphorylates FOXO and allows its translocation from cytoplasm to the nucleus (Yamaguchi *et al.* 2013). When activated, FOXO transcription factors inhibit tumour development and increase lifespan through an antioxidant activity and DNA repair mechanisms (Greer *et al.* 2007; Greer and Brunet 2007; Sedding 2008). FOXO transcription factors are linked to the mechanistic target of rapamycin pathway (mTOR), inhibited by insulin or insulin-like growth factor, but activated in the context of dietary restriction (Greer *et al.* 2007; Greer and Brunet 2007). Regarding nicotinamide, in addition to being involved in the absorption of vitamins (map04977), it is also part of the metabolic map 04212: longevity regulating pathway - worm. Accordingly, the oxidised form of nicotinamide dinucleotide (NAD+) activates histone deacetylases called sirtuins (Lin and Guarente 2003). Sirtuins have been shown to have a positive effect on longevity,

in particular by activating autophagy and slowing down mitochondrial activity (Boily *et al.* 2008; Anderson and Prolla 2009; Morselli *et al.* 2010; Someya *et al.* 2010). Here, the comparison between the most and the least senescent workers, respectively foragers and nest-workers, reinforces the link between metabolic activity, somatic maintenance and longevity. This link was already found in the comparison between queens and workers in our study (section 4.1), as well as in different contexts across several taxa in other studies (e.g. Kapahi and Zid 2004; Morselli *et al.* 2010; Kapahi *et al.* 2010; Liang *et al.* 2018).

*4.3. Foragers, a caste stressed, exposed to pathogens, and with larger amounts of saturated fatty acids*

We found 1,8-octanedioic to be in larger amounts in foragers than in nest-workers. This metabolite has been detected in the honey bee royal jelly and associated with metabolism activation (Terada *et al.* 2011; Li *et al.* 2013). A higher metabolic rate is associated with more oxidative stress, notably through the enhanced production of reactive oxygen species by the mitochondria (Sastre *et al.* 2000). Supporting the possibility that metabolic rates and oxidative stress are higher in foragers than nest-workers , up-regulated potential markers of oxidative stress were found in this caste. Prostaglandin F-2-alpha was detected only in foragers and another prostaglandin (5-Hexyltetrahydro-2-furanoctanoic acid) was up-regulated in foragers compared to nest-workers. In marine invertebrates, prostaglandins have been shown to help in acclimating to stressful environmental conditions (Brady 1983 and references therein). More prostaglandins in foragers might thus be expressed concomitantly or as a response to accumulating oxidative damages caused by higher metabolic activity or higher stress levels. Moreover, in support of higher oxidative stress levels in foragers, the forager caste was also the only one where we detected methyl5-hydroperoxy-6,8,9,11-bisepidioxy-12,14-eicosadienoate, a hydroperoxy fatty acid resulting from lipid peroxidation and involved in oxidative stress-related cell death in plants (Weber 2002).

When compared to nest-workers, foragers had a greater amount of two arachidonic acid derivatives (N - (5Z,8Z,11Z,15Z - eicosatetraenoyl) - alanine and N - hydroxy - 5Z,8Z,11Z,14Z - eicosatetraenoyl amine). As previously stated, arachidonic acid derivatives are precursors in the melanisation mechanism in response to bacterial infection. Moreover, 2S - amino - 3R,4R,5S - trihydroxy - 2 - (hydroxymethyl) – 14 – oxo – eicos - 6E - enoic acid, a.k.a sphingofungin E, was also only detected in foragers. As other sphingofungins, sphingofungin E has antifungal activity (Horn *et al.* 1992). Finding more potential immune metabolites in foragers agrees with the fact that they belong to the caste the most exposed to pathogens since they go outside to bring food back to the colony.

The classification-based PCA has shown that the class of saturated fatty acids was under-represented in queens, and Log2FC allowed to point out that almost all the saturated fatty acids were

found in greater amounts in foragers, whereas nest-workers had larger amounts of unsaturated fatty acids and unsaturated lysophosphatidylcholines. According to our data, it seems that saturated fatty acids characterize the castes with a faster ageing rate (workers and especially foragers) and unsaturated lipids the caste with a slower ageing rate (nest-workers *vs.* foragers). This opposes the usual positive association between lipid saturation level and longevity, found in several birds and mammals including humans (Puca *et al.* 2007), but also in the honey bee (Hulbert 2008). This positive association is presumably explained by the fact that saturated lipids are less prone to oxidative damage (Pamplona *et al.* 2002; Hulbert *et al.* 2007). Alternatively, the higher level of oxidative stress that is potentially due to high levels of unsaturated fatty acids might not affect ant workers, as already found in bee workers for protein and DNA damages (reviewed in Lucas and Keller 2014). Moreover, a lipid-specific analysis assessing the precise proportion of each kind of lipids would help to conclude about the exact composition of cell membrane in ant queens, foragers, and nest-workers.

*4.4. Communication, pheromones and other hormones*

In workers compared to queens, 9Z-octadecenamide was up-regulated. This compound is found in wasps, bees and ants to be a glandular secretion (do Nascimento *et al.* 1993; Calvello *et al.* 2003; Dani 2006; Billen *et al.* 2009). Because of its secretion in ant Dufour's gland, it might be an alarm or trail pheromone (do Nascimento *et al.* 1993). Because of their social role foragers and nest-workers use more often alarm or trail pheromones. Finding, higher quantity of such a pheromone underlines the link between behaviour and metabolome.

Foragers were the only caste in which we detected 2-alpha-Ethoxydihydrophytuberin and, compared to nest-workers, they had larger amounts of glucosyl (2E,6E,10x)-10,11-dihydroxy-2,6-farnesadienoate. These two sesquiterpenoids are precursors of two juvenile hormones that are crucial insect hormones involved in caste development (Hui *et al.* 2010; De Loof *et al.* 2015). In social insect workers, the juvenile hormone titer increases during the caste transition from nest-workers to foragers (Robinson and Vargo 1997; Elekonich *et al.* 2001; Dolezal *et al.* 2012), potentially under the control of vitellogenin (Guidugli *et al.* 2005), explaining why we found more precursors of the juvenile hormone in foragers.

*4.5. Intra-caste variations and non-identified functions*

Our analyses have shown metabolic differences not only between castes but also within castes. These differences could be seen on the PCA as well as on the clusters of the heat maps (**Figures 1-4)**. The PCA-based classification allowed us to highlight the classes responsible for these differences for both queens and workers (**ESM1** Table S5). Unfortunately, very little information in databases or the

literature was available for these metabolites. We were therefore able to describe them, but without providing a functional explanation. Although we were able to detect these intra-caste variations, it should be stressed that most of the variation is due to caste differences (**Figure 1** and **2**), for which we were able to provide functional explanations.

Albeit several metabolites or class of metabolites could not be linked to a particular function, it is worth noting that the overwhelming majority of the metabolites we detected are lipid derivatives, which may be inherent to sample preparation. The multiple biological roles lipid compounds fulfil could hence be highlighted: *e.g.* protection from desiccation and pollutants, mate recognition, energy fuel, key components of pheromones and sexual hormones (Stanley-Samuelson *et al.* 1988; Blomquist *et al.* 1991; Dani 2006). Surprisingly, we did not find any metabolite that appeared to be directly related to reproduction in queens, whereas proteins related to sperm motility were found in our previous proteomics study (Quque *et al.* 2019). Despite the relatively large initial data set (1991 metabolites),the proportion of metabolites that matched an annotation remained low (486/1991). In addition, among these annotated metabolites, not all could be associated with a specific function. It is therefore highly likely that some of these un characterised metabolites participate in the queen's reproductive function. This should encourage further studies to continue to describe the metabolites involved in the different functions of living organisms to reveal the explanatory power of metabolomics which, as our study shows, is capable of detecting fine differences between genetically related individuals.

**5. Conclusion**

This metabolomic analysis broadly confirmed the caste profiles established in our previous proteomics study (Quque *et al.* 2019) with less metabolites related to immune defence and oxidative damage in queens, but more of them in foragers, and more metabolites associated to digestion and nutrient assimilation in nest-workers. However, this study also shows that proteomics and metabolomics approaches are complementary. Indeed, we did not find reproduction-related metabolites in queens, while we found protein related to it. Conversely, nutrient sensing pathways linked to somatic maintenance and longevity were highlighted by the present metabolomics study but not by proteomics. The metabolomics analysis also underlined the prevalence of lipids in insect biological processes. We propose two possible improvements to this pioneering study. The first is to conduct the proteomic and metabolomic analysis jointly to guarantee a comparison of results 100% free of any possible sample and temporal bias. The second way of improvement would be to consider the fact that the division of labour in *L. niger* is mainly due to age. An experimental design studying the different castes at different ages would thus make it possible to differentiate the age-related changes in the metabolome and/or proteome from those purely caste-related.

**Author Contributions**

DH, FB, FC, CS and MQ designed the experimental protocol, MQ performed the behavioural observations and prepared samples before use in omics; DH prepared samples for metabolomics and performed the raw data processing; CV performed the LC-HRMS injections for metabolomics; MQ performed the whole statistical analysis and look for manual functional annotation of metabolites; FB retrieved KEGG functional information; MQ wrote the first draft; CV and DH wrote methodological parts of this draft; all authors edited the first draft.

**Data availability**

Supplementary data are available at https://ncloud2.zaclys.com/index.php/s/nLAdQ8Qrm43aAfi (Chapter 5) until publication.

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# Chapter 6| Combining metabolomics and proteomics to disentangle age and caste effects in black garden ant workers (*Lasius niger*)

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Sous les fleurs de cerisier
Grouille et fourmille
L'humanité.

Kobayashi Issa

Under the cherry blossoms
Abounds and swarms
Humanity.

Kobayashi Issa

**Abstract**

In social insects, workers perform distinct task according to the caste they belong to. Worker castes differ by different physiology and aging rates. In most species of ants, an individual is first a nest-worker performing tasks inside the nest, then, as it ages, become a forager bringing food to the colony. The caste shift thus seems inseparable from age, preventing from deciphering the role of caste and age in regulating the ant physiology. However, we set up an innovative protocol to obtain four groups of black garden ant (*Lasius niger*) workers different by age and caste: young foragers (Y.F), old foragers (O.F), young nest-workers (Y.NW) and old nest-workers (O.NW). Then, using mass spectrometry, we analysed both the proteome and metabolome of these four worker groups. The proteome-based classification sorted the samples only according to age, whereas the metabolome-based classification used caste as the main discriminating factor. This illustrates that the relative importance of age or caste may highly depend on the method used. Using log2 fold change comparisons showed that O.F were characterised by more oxidative stress, fewer antibiotic molecules and fewer metabolites involved in chemical communication. The younger ants, regardless of caste, had more metabolites and proteins related to xenobiotics detoxification and had a more active lipid metabolism than the older ones, which up-regulated the carbohydrate metabolism. The nest-workers, regardless of age, overexpressed two proteins related to the digestive system, confirming previous results. In addition, we detected a large quantity of metabolites with a potentially anti-cancer activity in all worker groups. Our study not only underlines how age and caste differently modulate worker ant's physiology, but it also points out few additional ageing mechanisms that may be conserved in very distant taxa (insects and rodents).

**Keywords:** social insects, division of labour**,** task specialization, eusociality, ageing, mass spectrometry, behaviour, metabolism, oxidative stress, cancer

## 1. Introduction

Certain insect species amongst bees, ants, wasps, and termites are remarkable for their well-established social structure within which each individual performs tasks specific to the caste to which it belongs. The division of labour has led to the naming of these insects as social insects. In recent years, many studies have shown that social insects are relevant models to understand the mechanistic basis of lifespans' diversity (i.e. ageing rate; Keller and Jemielity 2006; Parker 2010). For example, breeding individuals in social insects live up to 100 times longer than solitary insect species (Keller and Genoud 1997). However, the mechanisms usually known to be associated with ageing in mammals or birds sometimes show, if any, an opposite relationship in social insects. In the black garden ant (*Lasius niger*), telomeres are as long in workers as in queens, which live ten times longer (Jemielity *et al.* 2007). Similarly, genes coding for superoxide dismutases, an antioxidant enzyme family, are found to be more strongly expressed in female workers (Parker *et al.* 2004; Corona *et al.* 2005; Corona and Robinson 2006). Several contradictory studies on the accumulation of oxidative damage with age in insects underline the non-universality of the free radical ageing theory among the animal kingdom (Lucas and Keller 2014). Furthermore, caste membership determines the ageing rate of social insects. In the protected conditions of the laboratory, the major workers of the weaver ant *Oecophylla smaragdina* keep dying faster than the minors, indicating that the rate of ageing is caste-dependent, even within workers (Chapuisat and Keller 2002). Even more strikingly, it has been shown in the honey bee (*Apis mellifera*) that forcing foragers to resume performing tasks inside the nest, rather than foraging, leads to a restoration of memory capacities (Baker *et al.* 2012; Münch and Amdam 2013) and a reversal of immunosenescence (Amdam *et al.* 2005). The caste thus plays a predominant role in the ageing rates of social insects.

The shift from one caste to another can be favoured by external variables and adapt to variations in the environment (Herbers 1980; Gordon 1989; Yang *et al.* 2004; Warner *et al.* 2018), as well as it can adapt to variation in the needs during the colony maturation (Robinson 1992). Similarly, if a significant proportion of workers from one caste disappears (*e.g.* predation, weather, experimental manipulations), then some workers from other castes change caste and replace them (Gordon 1989; Robinson 1992; Konate *et al.* 2000; Münch *et al.* 2013). However, the most explanatory factor for caste change remains age, and this phenomenon is termed as age polyethism. It is observed in most species of social insects: the honey bee (Seeley 1982), most species of ants (except for example the primitive group of Amblyopones (Traniello 1978), wasps (O'donnell and Jeanne 1993; Naug and Gadagkar 1998; Shorter and Tibbetts 2009) and termites (see Soleymaninejadian *et al.* 2014 for a review of the different

polyethisms occurring in termites). In this context, it is therefore difficult to distinguish the respective influences of caste and chronological age on ageing rate.

Nevertheless, some studies have tried to disentangle the respective influences on ageing of these two parameters. To this end, they took advantage of the worker behavioural flexibility, capable of changing caste to compensate for the loss of individuals from another caste (old foragers resuming to within-nest tasks or nest-workers foraging earlier than expected). The details of such experimental protocols leading to four groups that differ according to the combination of age (young or old) and caste (nest-worker or forager) can be found with clear illustrations, for instance in honey bees (Münch *et al.* 2013), black garden ants (Dussutour *et al.* 2016), and *Temnothorax longispinosus* ants (Kohlmeier *et al.* 2018a). In honey bees, the caste-related behaviour predicts better than age the expression of genes (Whitfield *et al.* 2003) and the proteome of the antennae is independent of the age of the individual (Iovinella *et al.* 2018). On the contrary, the brood pheromone, involved in caste maturation, acts in a both age- and caste-dependent manner (Alaux *et al.* 2009). In the ant *Temnothorax longispinosus* (Kohlmeier *et al.* 2018a), workers express more genes (in whole organism) involved in behavioural differences than genes associated with age (ten times more) or fertility (50 times more). In particular, the nest-workers, called brood carers in this study, expressed more genes coding for vitellogenins, a family of proteins involved in caste differentiation in social insects (Kohlmeier *et al.* 2018b). Moreover, lipid metabolism, response to oxidative stress and DNA repair appeared more prominent in nest-workers compared to foragers. Conversely, despite significant behavioural and anatomical differences, transcriptomics comparison between queen and workers in *L. niger* showed that the divergence in gene expression were better explained by age than caste. Thus, there is so far no consensus on the respective importance of age and social caste effect on ageing parameters in social insects, with proteomic or genomic analyses being always conducted separately.

The aim of the present study is to complement our still sparse knowledge of the metabolic pathways controlled by the age and/or caste in social insects. To discriminate between the age and social effects, we successively sampled workers from colonies of the black garden ant (*Lasius niger*). The first sampling occurred at the time of colony foundation where both castes had young individuals. One year later, we sampled the same colonies a second time. As the eggs were regularly removed before hatching, ants of both worker castes were older. Only an individual follow-up throughout the worker's life would make it possible to guarantee that no caste shift has ever occurred. However, in the controlled laboratory conditions preventing ant colonies from major demographic changes, this protocol, contrary to former protocols, maximizes the likelihood to obtain old nest-workers that were never foragers and therefore

do not keep any potential molecular trace of past foraging activities. We then analysed on the same samples both the metabolome and the proteome of ants, to get two complementary pictures.

## 2. Methods

### *2.1. Ant keeping and set-up of experimental groups*

The black garden ant is a very common species in Western Europe where it predominantly inhabits urban habitats (Konorov *et al.* 2017). For this species, the absence of dimorphism between workers and the monogyny of the colonies reduce the potential sources of variation other than age and caste, which are of interest here. In laboratory conditions, black garden ant workers have been shown to live 310 days on average and up to 1094 days, *ca.* 3 years (Kramer *et al.* 2016). In our study, wild newly mated queen ants were collected in Lausanne, Switzerland (N 46.5234, E 6.5791). After being placed in individual glass tubes in the dark, 97 queens established a viable colony. Colonies were kept at a temperature of 21°C at night and 26°C during the day, relative humidity was 50-60%, the photoperiod mimicked the natural photoperiod of the capture area. Once a week, ant colonies were provided with a 0.3 M sugar water solution and mealworms.

After the first workers hatched, we let the colony develop for a month without any intervention, except feeding the ants. Due to the limited number of individuals available, young colonies at foundation sometimes have a less strict division of labour (Ferreira Brandao 1983; Holbrook *et al.* 2011). By waiting a month before the first collection, we allowed the colonies to grow sufficiently for the specialisation of tasks to take place. In addition, more developed colonies ensured that we could collect enough older individuals from the same colonies several months later. After a month, we ran the caste segregation protocol for the first time. The segregation of castes was based on their respective behaviours. Following a 48-hour period of starvation, colonies were proposed a 1 M sugar water. To maximize the forager recruitment, we waited five minutes after the first forager discovered the food source. Then, all the ants that came to the food source were considered as foragers, collected, and marked on the abdomen with an acrylic ink (Posca ©). We carried out this protocol three times with four days interval to ensure we captured all foragers and to allow the colony to rest. At the end of the procedure, not-marked ants were considered as nest-workers. These young nest-workers and foragers were flash frozen in liquid nitrogen and stored intact at -80°C until use. From that moment on, we followed the colonies carefully and removed the eggs in the last stages of maturation before they hatched. In this way, when we ran the caste segregation protocol for the second time, 11 months later, all the ants in the colonies were 11-12 months old. These two groups (nest-workers and foragers) of old workers were also flash frozen in liquid nitrogen and stored at -80°C until use. We ended up with four

worker groups of both different age and caste: young foragers (Y.F), old foragers (O.F), young nest-worker (Y.NW) and old nest-workers (O.NW).

Before use in mass spectrometry analyses (*i.e.* proteomics and metabolomics), ink on foragers' abdomens was removed with acetone. We constituted pools of 50 workers for each group and used 5 pools per group (250 workers). Pools are made of a balance mixture from different colonies to remove this possible confounding effect (2-3 workers of each colony). Ants were ground under liquid nitrogen for 1 min at 30 Hz with steel beads (Ø 2 mm, Mixer Mill MM400, Retsch, Eragny Sur Oise, France). Tubes containing the resulting powder and beads were then stored at -80°C until their use for mass spectrometry.

*2.2. Proteomics preparation*

Unless otherwise specified, all chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2.1. Sample Preparation

The frozen samples (powder) were suspended in 220 µL of lysis buffer (urea 8M, thiourea 2M, dithiothreitol [DTT] 1%, ammonium hydrogen carbonate [$HNH_4CO_3$] 0.1M, protease inhibitors 1.6 µM to 2 mM and sonicated on ice for 2 x 10 sec. at 135W, then samples were centrifuged (2000 x g, 2 min, RT) to eliminate possible cuticle remnants. Eight volumes of cold acetone (ThermoFisher Scientific, Rockford, IL, USA) were finally used for protein precipitation at -20°C overnight. Precipitated proteins were pelleted by centrifugation (13500 x g, 20 min, 4°C), washed one time with cold acetone and then dissolved in Laemmli buffer (Tris 10 mM pH 7.5, EDTA 1 mM [Fluka, Buchs, Switzerland], β-mercaptoethanol 5%, SDS 5%, glycerol 10% [ThermoFisher Scientific]). Sonication and centrifugation were repeated as above to pellet and eliminate possibly remaining cell debris. Acetone fractions were all evaporated using a vacuum centrifuge (SpeedVac, Savant, Thermoscientific, Waltham, MA, USA) then kept at -20°C for further metabolomics analysis (see section 2.3).

Total protein concentration was determined using the RC-DC Protein Assay kit (Bio-Rad, Hercules, CA, USA). At this stage, a reference sample comprising equal amounts of all protein extracts was made, to be analysed regularly during the whole experiment and allow QC-related measurements. Twenty µg of proteins from each sample were loaded onto SDS-PAGE gels (4% polyacrylamide for the stacking gel and 12% for the resolving gel) and electrophoresed for 20 minutes at 50 V then 20 minutes at 100 V. Proteins were thereafter fixed by a 15-minute incubation of gels in a solution composed of 50% ethanol and 3% phosphoric acid. Staining was performed using colloidal Coomassie Blue (30 min), and

visualization of proteins allowed five protein bands (2 mm each) to be excised from the gel. After destaining using acetonitrile/ammonium hydrogen carbonate 25 mM (75/25, v/v) and dehydration using pure acetonitrile, proteins were reduced and alkylated in-gel using 10 mM DTT in 25 mM ammonium hydrogen carbonate buffer (30 minutes at 60°C then 30 minutes at RT) and 55 mM iodoacetamide in 25 mM ammonium hydrogen carbonate buffer (20 min at RT in the dark), respectively. Gel slices were then washed using 25 mM ammonium hydrogen carbonate buffer (5 min, RT) and acetonitrile (5 min, RT) three times, and dehydration was finally performed using pure acetonitrile (2 x 5 min, RT). In-gel digestion of proteins was performed overnight at 37°C using trypsin (Promega Madison, WI, USA; 40 ng per band), and the resulting peptides were extracted twice (2 x 45 min) on an orbital shaker (450 rpm) using a solution composed of 60% acetonitrile and 0.5% formic acid in water. Another peptide extraction step was then performed (1 x 15 min) using 100% acetonitrile. At this stage, a set of reference peptides (iRT kit; Biognosys AG, Schlieren, Switzerland) was added to peptide extracts (6µL/sample after resuspension in 500mL of 20% acetonitrile/1% formic acid) for QC-related measurements. Organic solvent was thereafter evaporated using a vacuum centrifuge (SpeedVac) and the volume of peptide extracts was adjusted to 27 µL using a solution composed of 1% acetonitrile and 0.1% formic acid in water.

2.2.2. nanoLC-MS/MS analysis

NanoLC-MS/MS analysis was performed using a nanoUPLC-system (nanoAcquity; Waters, Milford, MA, USA) coupled to a quadrupole-Orbitrap hybrid mass spectrometer (Q-Exactive plus; Thermo Scientific, San Jose, CA, USA). The system was fully controlled by XCalibur software (v3.0.63; ThermoFisher Scientific). Samples (1 µl) were first concentrated/desalted onto a NanoEAse M/Z Symmetry precolumn (C18, 100 Å, 5 µm, 180 µm × 20 mm; Waters) using 99% of solvent A (0.1% formic acid in water) and 1% of solvent B (0.1% formic acid in acetonitrile) at a flow rate of 5 µl/min for 3 minutes. A solvent gradient from 1 to 6% of B in 0.5 minute then from 6 to 35% of B in 60 minutes was used for peptide elution, which was performed at a flow rate of 450 nL/min using a NanoEAse M/Z BEH column (C18, 130 Å, 1.7 µm, 75 µm x 250 mm; Waters) maintained at 60 °C. Samples were analysed randomly per block, each block being composed of one biological sample from each group. The reference sample was analysed six times throughout the experiment. In between each sample, washing of the column using 90% acetonitrile during 6 minutes and running of a solvent blank allowed limiting carry-over effects. Peak intensities and retention times of reference peptides were monitored in a daily fashion.

The Q-Exactive Plus was operated in positive ion mode with source temperature set to 250 °C and spray voltage to 1.8 kV. Full-scan MS spectra (300−1800 m/z) were acquired at a resolution of 70 000 at

m/z 200. MS parameters were set as follows: maximum injection time of 50 ms, AGC target value of $3 \times 10^6$ ions, lock-mass option enabled (polysiloxane, 445.12002 m/z), selection of up to 10 most intense precursor ions (doubly charged or more) per full scan for subsequent isolation using a 2 m/z window, fragmentation using higher energy collisional dissociation (HCD, normalised collision energy of 27), dynamic exclusion of already fragmented precursors set to 60 seconds. MS/MS spectra (300-2000 m/z) were acquired with a resolution of 17500 at m/z 200. MS/MS parameters were set as follows: maximum injection time of 100 ms, AGC target value of $1 \times 10^5$ ions, peptide match selection option turned on.

2.2.3. Mass spectrometry data processing

Raw data were processed using MaxQuant v1.6.7.0 (Cox *et al.* 2014) . Peak lists were created using default parameters. Using Andromeda search engine implemented in MaxQuant, peaklists were searched against a UniprotKb protein database (*Lasius niger*, TaxID 67767; 18217 entries) created in November 2019 with MSDA software suite (Carapito *et al.* 2014). The database was complemented by Andromeda with the addition of the sequences of common contaminants like keratins and trypsin (247 entries) and of decoy (reverted) sequences for all *Lasius niger* proteins. Parameters were set as follows: precursor mass tolerance set to 20 ppm for the first search and to 4.5 ppm for the main search after recalibration, fragment ion mass tolerance set to 20 ppm, carbamidomethylation of cysteine residues considered as fixed modification, oxidation of methionines and acetylation of protein N-termini considered as variable modifications, peptide length of minimum 7 amino acids, maximum number of trypsin missed cleavages set to one, false discovery rate (FDR) set to 1% for both peptide spectrum matches and proteins. The proteins identified with a single peptide or with a negative score were discarded from identification data, as well as decoy hits and potential contaminants.

Protein quantification was performed using the MaxLFQ (label-free quantification) option implemented in MaxQuant. Parameters were set as follows: "minimal ratio count" of one, "match between runs" option enabled using a 0.7-minute time window after retention time alignment, consideration of both unmodified and modified (acetylation of protein N-termini and oxidation of methionines) peptides for quantitative determination, exclusion of shared peptides. All other MaxQuant parameters were set as default. Finally, criteria for retained proteins were as follows: at least two unique peptides quantified, no more than two missing values per group. Proteins absent in given groups (*i.e.* not detected at all) but satisfying above-mentioned criteria for the other groups were also retained. Among quantified proteins, 19 were identified as "un characterised" (1.9% of all quantified proteins) for which we searched known homologous proteins in the Protostomia clade using BLAST searches (FASTA program v36; downloaded from fasta.bioch.virginia.edu/fasta_www2/fasta_down.shtml), and only the best hits were retained. To validate this procedure, we automatically extracted orthology

annotations and sequence domains of *Lasisus niger* un characterised proteins and of their homologues from the OrthoDB (Kriventseva *et al.* 2019) and InterPRO (Mitchell *et al.* 2019) resources. The relevance of the match between *Lasisus niger* un characterised proteins and their homologues was then checked manually. The mass spectrometry proteomics data will be deposited, prior to submission, to the ProteomeXchange Consortium via the PRIDE (Vizcaíno *et al.* 2016) partner repository. From QC-related measurements, we could see that the whole analysis system remained stable throughout the experiment. Indeed, a median coefficient of variation (CV) of 1.3% was calculated for retention times of iRT peptides over all injections, and a median CV of 28% was computed for all LFQ values obtained from the repeated analysis of the reference sample.

### *2.3. Metabolomics preparation*

#### 2.3.1. Chemicals

Deionised water was filtered through a Direct-Q UV (Millipore) station, isopropanol and methanol were purchased from Fisher Chemicals (Optima ® LC/MS grade). Deuterium labelled [$^2H_6$](+)-cis,trans-abscisic acid ($^2H_6$-ABA) (OlChemIm). NaOH was obtained from Agilent Technologies, acetic acid formic acid from Sigma Aldrich.

#### 2.3.2. Sample preparation

Samples used for proteomics were dried using a SpeedVac. The pellets were resuspended with 500µl ethyl acetate and 300µl water. The samples were vortexed for 10 seconds, after phase partitioning the ethyl acetate phase was harvested for each sample and stored until LC-MS/MS analysis. The water phase was collected from each sample and diluted with 1 ml water acidified with 1 % formic acid. The acidified water phase was then desalted using Solid Phase Extraction (SPE) based on HLB matrix Oasis 96-Well plate 30µm (5mg) Waters coupled to a vacuum pump. Each SPE well was conditioned with 1 ml methanol, than with 1 ml water. The samples were then applied on the SPE and washed with 1 ml acidified water with 0.1% formic acid. The samples were then eluted with 700µl methanol. The elution fractions were pooled to the ethyl acetate fractions and analysed in liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) described in the next section.

#### 2.3.3. LC-HRMS analysis

Samples were analysed using liquid chromatography coupled to high resolution mass spectrometry on an UltiMate 3000 system (Thermo) coupled to an Impact II (Bruker) quadrupole time-of-flight (Q-TOF) spectrometer. Chromatographic separation was performed on an Acquity UPLC ® BEH C18 column (2.1x100mm, 1.7µm, Waters) equipped with and Acquity UPLC ® BEH C18 pre-column (2.1x5mm, 1.7µm, Waters) using a gradient of solvents A (Water, 0.1% formic acid) and B

(MeOH, 0.1% formic acid). Chromatography was carried out at 35°C with a flux of 0.3mL.min$^{-1}$, starting with 5% B for 2 minutes, reaching 100% B at 10 minutes, holding 100% for 3 minutes and coming back to the initial condition of 5% B in 2 minutes, for a total run time of 15 minutes. Samples were kept at 4°C, 10µL were injected in full loop mode with a washing step after sample injection with 150µL of wash solution ($H_2O$/MeOH, 90/10, v/v). The spectrometer was operated in positive ion mode on a mass range of 20 to 1000 Da with a spectra rate of 2Hz in AutoMS/MS scan mode. The end plate offset was set at 500 V, capillary voltage at 2500 V, nebulizer at 2 Bar, dry gas at 8 L.min$^{-1}$ and dry temperature at 200°C. The transfer time was set at 20-70µs and MS/MS collision energy at 80-120% with a timing of 50-50% for both parameters. The MS/MS cycle time was set to 3 seconds, absolute threshold to 816 cts and active exclusion was used with an exclusion threshold at 3 spectra, release after 1 min and precursor ion was reconsidered if the ratio current intensity/previous intensity was higher than 5. A calibration segment was included at the beginning of the runs allowing the injection of a calibration solution from 0.05 to 0.25min. The calibration solution used was a fresh mix of 50mL isopropanol/water (50/50, v/v), 500µL NaOH 1M, 75µL acetic acid and 25µL formic acid. The spectrometer was calibrated in high precision calibration (HPC) mode with a standard deviation below 1ppm before the injections for each polarity mode, and re-calibration of each raw data was performed after injection using the calibration segment.

2.3.4. Metabolite identification and quantification

Raw data were processed in MetaboScape 4.0 software (Bruker): molecular features were considered and grouped into buckets containing one or several adducts and isotopes from the detected ions with their retention time and MS/MS information when available. The parameters used for bucketing were a minimum intensity threshold of 10000, a minimum peak length of 4 spectra, a signal-to-noise ratio (S/N) of 3 and a correlation coefficient threshold set at 0.8. The $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ ions were authorised as possible primary ions. Replicate samples were grouped and only the buckets found in 80% of the samples of one group were extracted from the raw data. The obtained list of buckets was annotated using SmartFormula to generate raw formula based on the exact mass of the primary ions and the isotopic pattern. The maximum allowed variation on the mass (⌀m/z) was set to 3ppm, and the maximum mSigma value (assessing the good fitting of isotopic patterns) was set to 30. To put a name on the obtained formulae, analyte lists were derived from Human Metabolite Database (HMDB, hmdb.ca), FooDB (foodb.ca), LipidMaps (lipidmaps.org) and SwissLipids (swisslipids.org). The parameters used for the annotation with the analyte lists were the same as for SmartFormula annotation.

*2.4. Statistics and biological interpretation of mass spectrometry results*

Unless otherwise specified, the analysis and graphical representations were made using R software, version 4.0 (R Core Team 2019).

2.4.1. Datasets

We only analysed the proteins and metabolites (grouped under the term of 'analytes'), which we were sure to be present or absent in a given group. Analytes were considered present for sure in a group only when present in at least 3 out of 5 samples of this group. Conversely, analytes were considered completely absent from a group only when none of the samples contained the analyte. Consequently, analytes present in only 1 or 2 samples of a group were discarded from the statistical analysis. For analytes not present in all samples of a given group but in 3 or 4 out of 5 samples, we imputed the missing values using an iterative PCA (principal component analysis) algorithm (MissMDA package v.1.17, Josse and Husson 2016). Considering only the proteins and metabolites present or absent for sure in a group, missing data represented 2.3 % of the proteomics data and 0.9 % of the metabolomic data. In the proteomics statistical analysis, we discarded one young forager sample and one young nest-worker sample identified as outliers during the statistical workflow. In all statistical analyses, proteins and metabolites were studied separately to better underline their respective effects on the physiology of ant workers.

2.4.2. Heat maps and pairwise comparisons

First, we verified whether the relative abundance of analytes in the different groups could discriminate them from each other and whether the discrimination criterion was age or caste. For this, we used the 'rlog' function of the DESeq2 package (v.1.28, Love 2014) to transform the proteomics and metabolomics data to the log2 scale in a way which minimizes differences between samples with small counts, and normalises with respect to the size of the dataset. Based on this normalised data, we built heat maps with the ComplexHeatmap package (v.2.42, Gu *et al.* 2016). Heat maps represent the most differentially express metabolites and perform a hierarchical clustering, that reflect the proteomic or metabolomic similarities between samples. Then, we sought to identify exactly which proteins and which metabolites were expressed depending on the age or caste. Comparing O. NW vs. O.F or Y.NW vs. Y.F provides information on the influence of caste since the age between the groups compared is identical. Conversely, keeping the caste constant, by comparing Y.NW vs O.NW or Y.F vs O.F, makes it possible to assess the influence of age. We identified the analytes that differed most strongly between the groups compared two-by-two by calculating log2 fold-changes (further referred to as log2FC) of each analyte with the DESeq2 package. In this analysis, we retained only the analytes with a false

discovery rate (FDR) lower than 0.05 and a log2FC higher than 2 (up-regulated) or lower than -2 (down-regulated).

2.4.3. Classification and functional annotation of metabolites

To understand the biological meaning of the proteomics and metabolomics profiles characterised by the pairwise comparisons, we automatically retrieved metabolic maps from the KEGG database (genome.jp/kegg) when available. If not, we "manually" looked in the literature for functions fulfilled by the molecules in concern.

Metabolites may encompass very distinct molecules: *e.g.* lipids, free amino acids, free nucleic acids, carbohydrates. We automatically classified metabolite thanks to the ChemRICH online tool (chemrich.fiehnlab.ucdavis.edu; Barupal and Fiehn 2017). We used these classes of metabolite in a principal component analysis (PCA) to see whether some groups of metabolites characterised rather the caste than age, and conversely (FactoMineR package v.2.3, Lê *et al.* 2008). Using the PCA coordinates, we calculated the intra-group repeatability (package "rptR" v.09.22, Stoffel *et al.* 2017) to assess their homogeneity. The metabolite's class was also added to heat maps to provide additional information about the most segregating metabolites.

**3. Results**

The combined mass spectrometry analyses (LC-MS/MS) characterised 712 metabolites and 1006 proteins among our four experimental groups of workers in the *Lasius niger* ant. Regarding metabolites, 203 were automatically named (28%). These putative annotations mainly came from the Human Metabolome Data Base (HMDB). For the 20 proteins that did not match any known sequence in *Lasius niger* ('un characterised proteins'), the search for homologous proteins in the Protostomia clade provided us with three additional reliable annotations. Therefore, 16 proteins out of 1006 quantified (1.6%) were still uncharacterised. Original data sets are available in electronic supplementary material (**ESM1**, Table S1-S3). The tables used for the log2FC analysis, cleaned from metabolites or proteins found in less than 3/5 samples in a group, are available in ESM1, **Tables S4 and S5**. The statistics summary (log2FC, FDR, class, biological processes) of these analyses are available in **Tables S7 and S8**, as well as the references used to link analytes to the biological processes mentioned below.

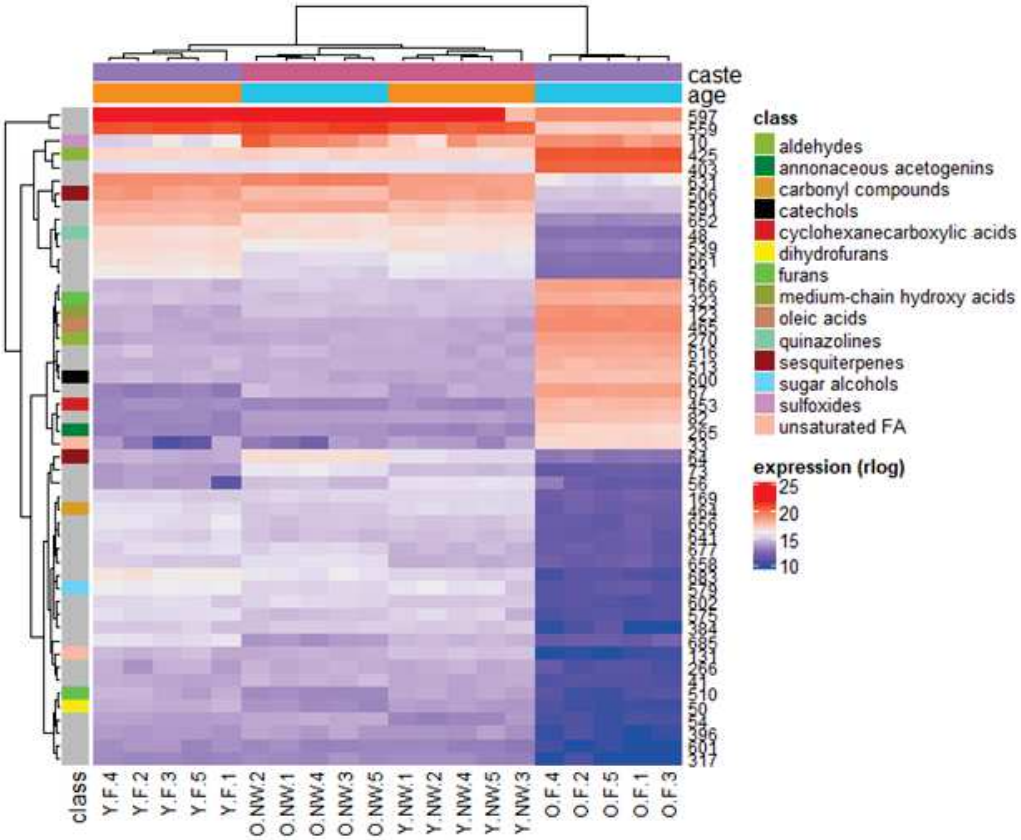


**Figure 1. Heat map of the 50 most expressed metabolites amongst our four experimental groups in *L. niger*.** At the top, colours indicate the age (orange: young, light blue: old) and caste (pink: nest-workers, purple: foragers). The left column indicates the metabolite class. Grey means a NA value. At the bottom are the sample identifiers (F: forager, NW: nest-worker, Y: young, O: old). For a given metabolite, the right column indicates its ID rather than the full name for legibility reasons (correspondence is indicated in every table provided). All metabolites presented here have an FDR < 0.05 and |log2 FC| > 2. In the caption, FA stands for fatty acids.

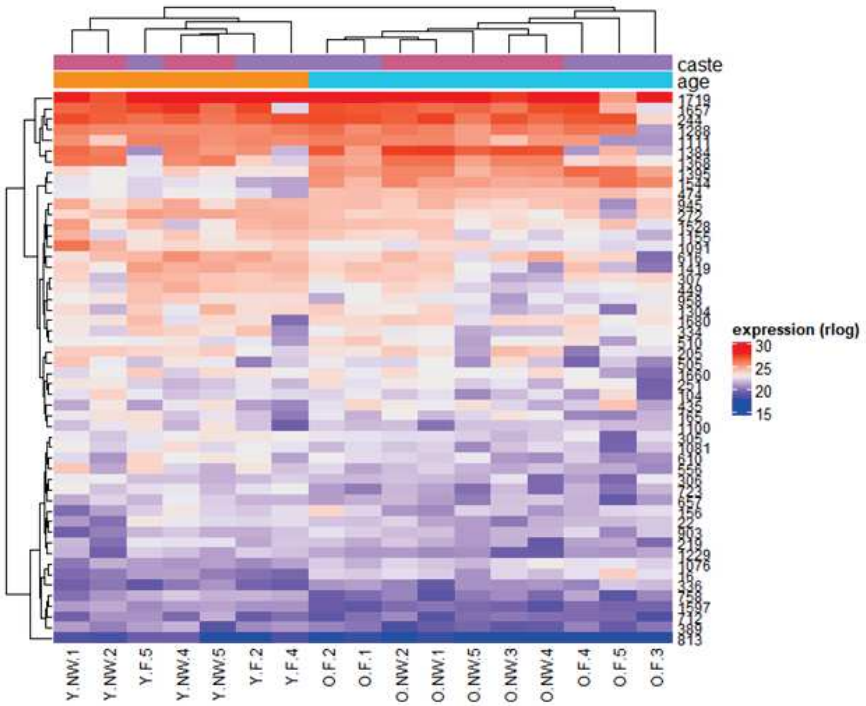


**Figure 2. Heat map of the 50 most expressed proteins amongst our four experimental groups in *L. niger*.** At the top, colours indicate the age (orange: young, light blue: old) and caste (pink: nest-workers, purple: foragers). At the bottom are the sample identifiers (F: forager, NW: nest-worker, Y: young, O: old). For a given protein, the right column indicates its ID rather than the full name for legibility reasons (correspondence is indicated in every table provided). All proteins have an FDR < 0.05 and |log2 FC| > 2.

*3.1. Absent metabolites*

Regarding the analytes completely absent from at least one caste, they were mainly found absent in old foragers, but their unspecific role prevented us from attributing any associated biological meaning. Consequently, absent proteins and metabolites will not be further discussed thereafter.

*3.2. Hierarchical clustering and heatmaps*

We drew two heat maps to picture both the clustering obtained with metabolomics and proteomics data. We added a colour code to precise the class of metabolites on the concerned heat map. The metabolomics-based clustering (**Figure 1**) shows that the four experimental groups have a weaker intra-group variation than inter-group variation and the O.F group differs the most when compared to the three other groups. But then, the two groups of nest-workers (Y.NW and O.NW) are closer to each other than to Y.F, indicating that the caste has a stronger impact than age on metabolites. On the contrary, the proteomics-based clustering (**Figure 2**) assorts samples according to individual's age.

*3.3. Pairwise comparisons involving age differences*

When comparing Y.F to O.F, we found larger amounts of one metabolite linked to amino acid metabolism (2-isopropylmaleate) and of two additional metabolites related to glycerophospholipid metabolism (lysoPE(0:0/18:1(11Z)) and lysolecithin). Y.F over-expressed some nutrition-related metabolites: two linked to protein metabolism (N-acetylputrescinium and N-methyltyramine), one to vitamin K (1,4-dithiothreitol) metabolism, and one to nucleic acid metabolism (nebularine). However, Y.F up-regulated many more lipid metabolism-related metabolites (eight), especially unsaturated fatty acids used as energy source (*e.g.* (1RS,2RS)-guaiacylglycerol 1-glucoside, 14,16-nonacosanedione, 6,8-tricosanedione). The assimilation and metabolism of lipids in general, not only unsaturated fatty acids, was over-represented in Y.F (*e.g.* ximenic acid, (E)-2-tetracosenoic acid, 9-Decenoylcarnitine) and two of these lipids have known antibiotic activity (armillarin, cyclohexaneundecanoic acid). Many other antibiotic metabolites were found more abundant in Y.F: *e.g.* 13-eicosenoic acid, 3-ethyl-5-methyl-1,2-cyclopentanedione, and thiophene. Besides, Y.F over-expressed metabolites linked to xenobiotics degradation (1-methoxyphenanthrene and cyclohexanone) and several compounds involved in chemical communication (*e.g.* tetracosanedioic acid, oleamide, hexadecanedioic acid, 2-[(methylthio)methyl]-2-butenal ). Only one compound potentially involved in chemical communication was found more abundant in O.F: octanal, an oleic acid. While O.F showed up-regulation of four metabolites associated to oxidative damage (*e.g.* 3,4-dimethoxybenzoic acid, robustocin), Y.F had only one up-expression of such metabolites (psilostachyin). Two metabolites with a potential anti-inflammatory and/or anti-tumour activity were more abundant in O.F ([6]-shogaol, chrysophanol-9-

anthrone) and three metabolites with similar properties in Y.F (*e.g.* armillarin, psilostachyin and thiophene).

The proteome analysis confirmed the metabolome analysis by finding proteins up-regulated in Y.F *vs.* O.F related to xenobiotic detoxification (cytochrome p450 4g15), immunity (transferrin), vitamin metabolism (alpha-tocopherol transfer), lipid metabolism (acetyl-cytosolic), and protein synthesis (glycyl-tRNA synthetase and peptidyl-prolyl cis-trans isomerase). O.F also over-expressed proteins related to protein synthesis when compared to Y.F. However, contrary to Y.F, they up-regulated many proteins involved in carbohydrate metabolism (*e.g.* glucosidase, maltase 1, trehalose transporter tret1-like) but no protein related to lipid metabolism. Finally, O.F also had greater amounts of two proteins involved in cell structure, motility, proliferation, and adhesion (protein g12 and tetraspanin).

No metabolite up- or down-regulated in Y.NW *vs*. O.NW was found. However, the proteomics analysis highlighted up-regulation of cuticular proteins (apd-3-like protein and cuticle isoform x1) or involved in xenobiotics detoxification (cytochrome p450 4g15) in Y.NW, and down-regulation of proteins involved in carbohydrate metabolism (alpha-like protein) and cell structure, motility, proliferation and adhesion (protein g12 and tetraspanin).

*3.4. Pairwise comparisons involving caste differences*

The comparison of Y.NW vs. Y.F showed that Y.NW up-regulated two metabolites linked to lipid metabolism (lysolecithin and lysoPC(16:1(9Z)), one involved in oxidative stress response (lysoPE(0:0/18:1(11Z))), and one involved in sulfur metabolism (dimethyl sulfoxide). The proteins up-regulated in Y.NW were associated with protein degradation (lysosomal aspartic protease) and midgut regeneration (arylphorin subunit alpha). No metabolite or protein was found down-regulated in Y.NW when compared to Y.F.

When compared to O.F, O.NW were found to have many more up-regulated metabolites in connection with lipid metabolism (*e.g.* (9Z,11E)-(13S)-13-hydroperoxyoctadeca-9,11-dienoate, Ceramide (d18:1/16:0), chemical communication (*e.g.* 3-hydroxytetradecanoic acid, 1,3-di-tert-butylbenzene) and immunity (*e.g.* thiophene, nebularine). O.NW also up-regulated one metabolite found in xenobiotic detoxification pathway (cyclohexanone) and one involved in protein K metabolism (1,4-dithiothreitol). Different metabolites exhibiting anti-cancerous, anti-inflammatory and anti-oxidant properties were found in larger amounts in both O.NW (*e.g.* psilostachyin, thiophene, alpha-terpinyl acetate) and O.F ([6]-Shogaol), and this was also true for metabolites involved in amino acid metabolism, such as 2-

isopropylmaleate (up in O.F) and n-methyltyramine (up in O.NW). The oleic acid octanal, already found typical of O.F when compared to Y.F (see above), was here typical of O.F when compared to O.NW.

The analysis of the proteome showed an up-regulation in O.NW vs O.F of proteins involved in lipid storage (lipid storage droplets surface-binding protein 2) and midgut regeneration (arylphorin subunit alpha). No protein was found down-regulated in O.NW compared to O.F.

3.5. *Classification-based PCA of metabolites*

After grouping the metabolites according to the class they belong to, we used a PCA to see if a particular class of metabolites was typical of a given experimental group. The first principal component (PC1) of this PCA explained 77.9 % of the variance and frankly split the old foragers (O.F) from the three other groups: young foragers (Y.F), young nest-workers (Y.NW) and old nest-workers (O.NW), see **Figure 3**. PC2 explained 14.1% of the variance and low PC2 values characterised nest-workers (both young and old ones), while higher values of PC2 characterised Y.F. PC3 only explained 4.1% of the variance. Consequently, PC3 and the next principal components were not considered in our analysis. The retained metabolite classes ($|r| > 0.5$ and $\cos^2 > 0.8$), their correlation coefficient, and contribution to axes of the PCA can be found in Electronic Supplementary Material (**ESM1**, Table S6). As it can be seen from PCA plots, the intra-group variation was low whatever the PCA axis considered (R = repeatability ± SEM [CI95%], p-value): PC1 (R = 0.996 ± 0.024 [0.948, 0.999], p = 0.001), PC2 (R = 0.989 ± 0.055 [0.833, 0.997], p = 0.001), and PC3 (R = 0.942 ± 0.129 [0.517, 0.987], p = 0.001). The experimental groups were thus well distinct from each other and very similar within them.

The O.F were characterised by the overexpression of only a few metabolite classes (12), *e.g.* medium chain hydroxy acids, oleic acids, and furans, whereas almost 30 additional classes, *e.g.* terpenoids, acyl carnitines, polyunsaturated alkamides, very long chain fatty acids were under-expressed (**Figure 3,** PC1). Nest-workers and especially O.NW, compared to Y.F had more benzene derivatives, long chain hydroxy acids, monoterpenoids, lysophosphatidylcholines and acyl carnitines; but less unsaturated fatty acids, phenanthrenes and derivatives, thiophenes, thiazoles and diterpenoids (**Figure 3**, PC2).

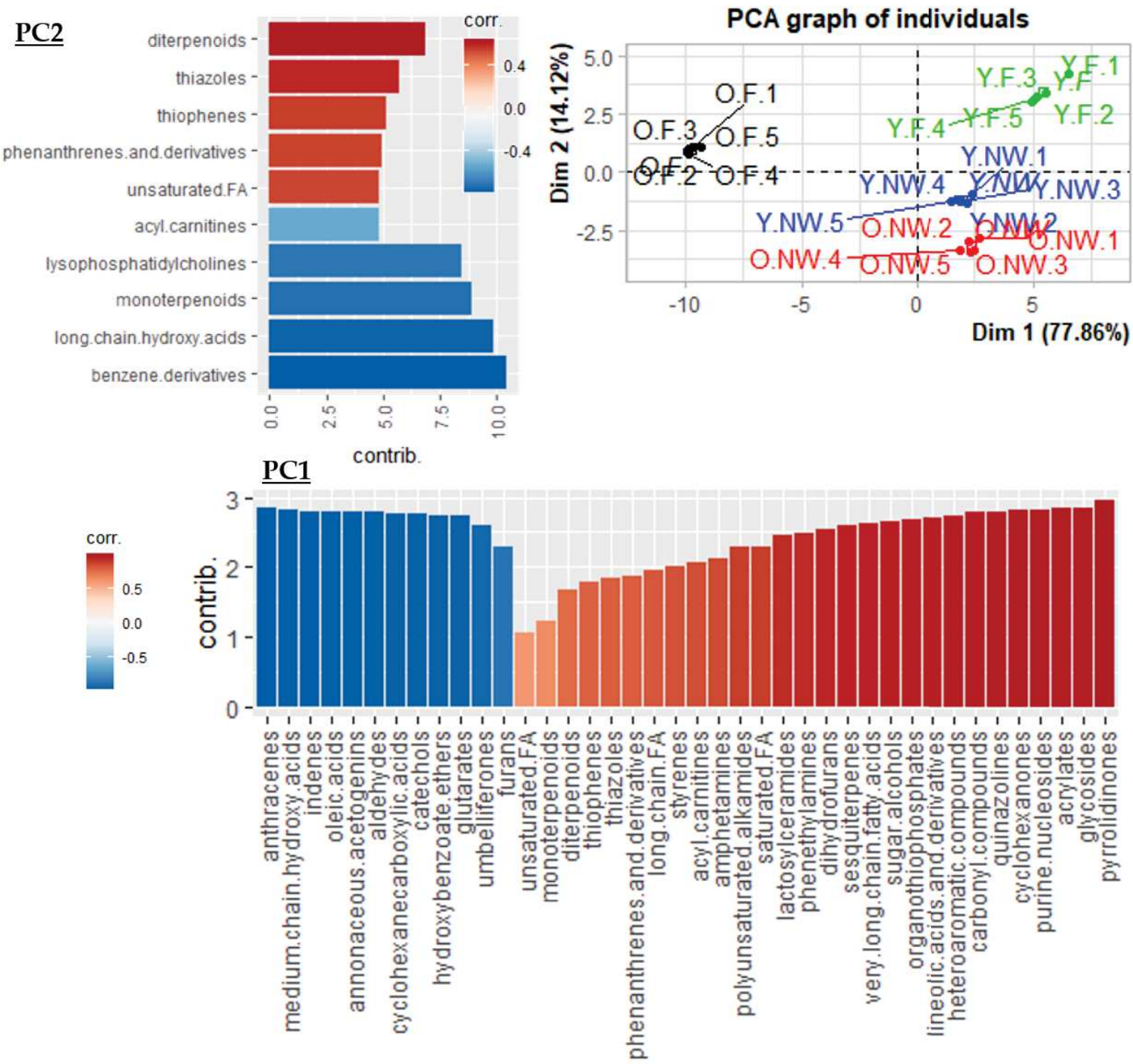


**Figure 3. Classification-based PCA (PC1 and PC2).** We ran a PCA with the metabolite classes as variables and explore the metabolomic differences amongst our four experimental groups in *Lasius niger* ants: **Y.F** (in green) = young foragers, **O.F** (in black) = old foragers, **Y.NW** (in blue) = young nest workers, **O.NW** (in red) = old nest-workers. The bar plots represent the classes of metabolites and their correlation with the first and second principal component (PC1 and PC2) from blue (negatively correlated) to red (positively correlated), as well as their contribution (bar length). All metabolites showed are correlated more than 50% to one of the two axis and $cos^2 > 0.8$. The list of metabolites is available in Table S6 (**ESM1**). *FA stands for fatty acids.*

## 4. Discussion

The purpose of this study was to disentangle the influence of caste and age on the physiology of workers from a social insect thanks to four experimental groups of ants that differed in age and caste: young nest-workers (Y.NW), young foragers (Y.F), old nest-workers (O.NW) and old foragers (O.F). We analysed the proteome and metabolome from the same samples for each individual. The combined results of these two approaches highlighted eight major biological processes expressed in a caste- and/or age-dependent manner: anti-cancer activity, immunity, detoxification of xenobiotics, digestion, cell dynamics, chemical communication, lipid and carbohydrate metabolisms and oxidative balance. Below we discuss these biological processes in the context of caste and age modulation of ant's phenotype.

### *4.1. The interaction between age and caste sets the old foragers apart*

Both the classification-based PCA (**Figure 3)** and heat maps (especially **Figure 1**) clearly illustrate that the phenotype of O.F is the most differentially expressed when compared to the three other groups. Thereafter, we assess the respective influence of caste (comparison to O.NW) and age (comparison to Y.F) in shaping the specificity of O.F.

First, regarding analytes with an antibiotic activity in O.F., we found nine metabolites and one protein up-regulated in Y.F, and six metabolites up-regulated in O.NW with such an activity. Thus, both age and caste are involved in the lower abundance of molecules related to immunity in O.F.

A similar interaction between age and caste was observed with chemical communication. Indeed, when comparing the quantities of metabolites linked to it, O.F always had less chemical signals than O.NW (caste effect) or Y.F (age effect), with the exception of one class of lipids, oleic acid and derivatives. This result therefore seems to indicate that O.F communicate much less with their congeners than do the other groups. Since O.F overexpressed few additional molecules (**Figure 3**), it suggests that the up-regulation of those derivatives of oleic acids may serve a specific biological function, and we can make a second hypothesis to explain the scarcity of communication-related molecules in O.F. Oleic acid, linoleic acid and their derivatives are known to be secreted in a very conservative manner within insect taxa, including ants, when they die (Buehlmann *et al.* 2014; Qiu and Cheng 2017; Sun *et al.* 2018). It is known that this signal helps ants to recognise a corpse and is involved in corpse management behaviours (Gordon 1983; Sun and Zhou 2013). Studies in social insects have shown that the chemical cues of death can change over time and trigger distinct behaviours depending on the social context and the species; *e.g.* removal, cannibalism, burial (Gordon 1983; Diez *et al.* 2013; Sun *et al.* 2017, 2018). It is also known that ants practice social immunity, for example, by reducing interactions with an individual recognised as sick (Cremer *et al.* 2007; Stroeymeyt *et al.* 2018). It would therefore be possible for members of the colony to detect senescent individuals *via* oleic acids. This may allow behavioural adaptions of social interactions, *e.g.* avoiding interactions with O.F, potentially

carrying more germs, in accordance with the fact that they have been shown in this study to have fewer antibiotic metabolites. It has also been shown that sick ants isolate themselves before dying (Heinze and Walter 2010; Bos *et al.* 2012). Both cases would lead to the social isolation of this group and therefore to a weaker synthesis of communication molecules.

Considering immunity and chemical communication, caste-related and age-related mechanisms seem to interact in the shaping of the complete individual phenotype of an individual. In the next two sections, we address mechanisms that appear to be preferentially regulated either by age or caste.

*4.2. Multiple processes depending on biological age*

Two pathways clearly influenced by age, regardless of the caste being compared (Y.F vs O.F or Y.NW *vs.* O.NW), were the carbohydrate and lipid metabolisms. Indeed, younger ant workers had more proteins and metabolites linked to lipid metabolism than older ones. On the other hand, old workers up-regulated proteins and metabolites related to carbohydrate metabolism. A previous study highlighted a change in whole body fat content in relation to probability of survival in ant workers under stress: more fat enhancing survival prospects (Dussutour *et al.* 2016). In the honey bee, the greater longevity of queens was proved positively associated with a low polyunsaturated lipids/monounsaturated lipids ratio (Haddad *et al.* 2007). A similar relationship has been shown in long-lived bird and mammals (Pamplona *et al.* 2002; Hulbert *et al.* 2007). All these observations suggest that lipid metabolism is crucial in determining either survival or lifespan. Our study rather supports this hypothesis, and underlines a metabolic shift with age, from lipid to carbohydrate use in ant workers. Whether this is a side-effect accompanying other functional changes with age remains to be determined. For instance, immunity or chemical communication were expressed more by the Y.F. than by the O.F, and these functions are lipid consuming (Gilbert 1967; Stanley-Samuelson *et al.* 1988; Lockey 1988; Sinclair and Marshall 2018), which could be reflected in enhanced lipid metabolism in Y.F. Whether the observed metabolic shift has a causal effect on worker's ageing rate, for instance *via* deleterious impact of carbohydrate use as fuel (Sun *et al.* 2014; Cooper *et al.* 2018; Xu *et al.* 2019), needs further experimental studies.

Compared to O.NW, Y.NW had greater quantities of cytochrome P450 4g15, belonging to the CYP4 sub-family of cytochrome P450. CYP4 have been found to be linked to the metabolism of toxic xenobiotics (Snyder 1998; Feyereisen 1999; Won *et al.* 2013). Similarly, Y.F, compared to O.F had greater quantities of this same protein, as well as two metabolites involved in the degradation of xenobiotics: cyclohexanone and 1-methoxyphenanthrene (KEGG maps 00930 and 00624). The capacity to detoxify the organism therefore may decrease with age in *Lasius niger* workers, which might be one of the strong mechanisms explaining survival rate over time.

Two proteins involved in cytoskeletal maintenance, proliferation and cell adhesion have also been systematically found expressed in an age dependent manner: protein G12 and tetraspanin (Dhanasekaran and Dermott 1996; Maecker *et al.* 1997; Hemler 2005). As far as we know, the role of these particular proteins in ageing has not yet been investigated. However, modification of the actin cytoskeleton has been linked to many ageing mechanisms such as apoptosis, protein aggregation, mitochondrial ROS production (Gourlay and Ayscough 2005). Plus, senescent cells show a decrease in proliferation and migration associated with cytoskeleton alterations (Nishio and Inoue 2005). The two proteins underlined by our analysis could thus be markers of cell senescence in older ant workers.

Finally, pairwise comparisons based on log2FC of proteins and metabolites associated with oxidative stress or response to oxidative stress highlighted age differences. Four metabolites were found more abundant in O.F (*vs.* Y.F), and only one less abundant. This result suggests that oxidative damage could accumulate over time in foragers. This result feeds the contradictory debate between studies showing an accumulation of oxidative stress with age in social insects and others showing the opposite (Lucas and Keller 2014). Since our study was exploratory, *i.e.* not specifically targeting oxidative balance markers, our results should lead to reconsider the way we classically assess oxidative stress in insects to find more relevant markers in them. Moreover, fighting against this additional oxidative stress has most probably an energetic impact on O.F, which would then have less energy to invest in xenobiotics detoxification and immunity, explaining the former results we found in O.F (above and section 4.1).

*4.3. A dependent caste digestive function*

When comparing the proteome of nest-workers with that of foragers (Y.NW *vs.* Y.F and O.NW *vs.* O.F), we systematically found the protein arylphorin subunit alpha in larger amount within nest-workers, independently of age. This protein stimulates the midgut stem cells and notably allows its regeneration after stress (Blackburn *et al.* 2004; Hakim *et al.* 2007; Castagnola *et al.* 2017). The importance of digestive function in *Lasius niger* nest-workers seems strong since we already found it in two previous studies (using proteomics (Quque *et al.* 2019) and metabolomics (Quque et al., *unpublished data*)). To our knowledge, no other study experimentally addressed this question in adult nest-workers. Two hypotheses can be formulated to explain this phenotype: (i) the excess of food is stored and pre-digested by nest-workers to make it quickly available for the rest of the colony in case of a food shortage; (ii) this pre-digested food would allow queens to process food without energy investment in their own digestive system. The mechanism proposed in the latter hypothesis would form an additional trade-off solution explaining the unexpected equation of high reproduction and high longevity in ant queens.

*4.4. Eusocial species, all immune to cancer?*

Already in the late 60's, 80% of the literature about cancer in insects focused on Drosophila (Harshbarger and Taylor 1968). Nowadays this proportion has hardly changed, perhaps even increased. Nevertheless tumour occurrence has been reported in some insects: *e.g.* Drosophila, cockroaches, migratory locust (Harshbarger and Taylor 1968; Tascedda and Ottaviani 2014; Aktipis *et al.* 2015) and we assume that insect have evolved protective mechanisms. We observed that the four experimental groups homogeneously express metabolites with potential anti-carcinogenic activities, notably by triggering cell cycle arrest or apoptosis (see ESM1, **Table S8**). Would those metabolites indicate that ants are immune to cancer? Such an ability has already been documented in another eusocial species, the naked mole rat. Studying this very long-lived rodent (32 years in laboratory) has conducted to show that their cancer resistance stems from multiple mechanisms not relying on replicative senescence but on early-acting tumour suppressor mechanisms: a strong early contact inhibition activated by hyaluronan, high-fidelity protein synthesis, more effective proteolysis by the proteasome system and through autophagy, a more active antioxidant barrier, and additional levels of cell cycle control (Seluanov *et al.* 2018). Smaller animals have fewer cells and therefore a lower probability of developing a tumour. The selection pressure on replicative senescence is therefore less strong in these species, which can afford long telomeres while maintaining a low risk of tumour (Seluanov *et al.* 2008; Gorbunova and Seluanov 2009). This risk is even lower if the animal has a short life span, but it can become non-negligible when the life span extends. This is the reason why small long-lived species such as social insects, at least the long-lived castes, are expected to develop anticancer mechanisms as described above in the naked mole rat. Although all groups expressed anticancer-linked metabolites, they were not identical between groups and a closer look at those putative anti-cancerous molecules highlighted group-dependent patterns. For example, the metabolites found in greater quantities in old foragers (O.NW vs. O.F and Y.F vs. O.F) can also be associated with the oxidative balance, those found in greater quantities in O.NW and Y.F (vs. O.F) with antibiotic functions, and those found in greater quantities in Y.NW (vs. Y.F) with glycerophospholipid metabolism. It would therefore be possible that the observed up-regulation of anti-cancer molecules rather co-varied in relation to the up-regulated functions described above, the anti-cancer activity being, in this case, only a side effect. We need dedicated experiments to answer this question. Given the extreme longevity of *L. niger* queens, 28 years in the laboratory (Keller 1998), it is to be expected that they will also develop means of prevention against the development of tumours. Although the potentiality of insect molecules for cancer therapy in humans have been investigated (Chernysh *et al.* 2002; Slocinska *et al.* 2008; Tonk *et al.* 2016), we found no study assessing their effectiveness in insects.

*4.5. The benefits of a double omics approach*

The combined use of proteomics and metabolomics appeared to be powerful in two aspects. First, it reinforced our conclusion by confirming that some of the pathways were modulated both in their proteomics and metabolomics signatures. For example, we found more antibiotic metabolites in young foragers than in older foragers (Y.F *vs*. O.F) and the protein transferrin, which holds an immune role (Geiser and Winzerling 2012), showed the same regulatory pattern. Secondly, the dual omics approach highlighted the variation of biological pathways that would not have been found if we had used only metabolomics (no metabolites linked only to digestion) or proteomics (no anti-cancer protein). Furthermore, the use of these two methods reinforced the idea that the phenotype of worker ants was both caste- and age-dependent. Indeed, metabolome-based clustering first highlighted the caste as the main discriminating factor among experimental group phenotypes, and then age as a secondary factor, whereas the proteome-based clustering underlined only the age factor. This dependence of the results on the omics approach may be due to the fact that the metabolome and proteome have different dynamics of temporal variation. Indeed, the proteome is modified over a longer period and depends partly on genome expression, whereas the metabolome responds more quickly to environmental variations. Thus, chronological ageing, which is a progressive process, dependent on the expression or inhibition of certain genes and proteins, would be preceded by a metabolic signature. Behavioural responses which may be more immediate, could be more rapidly reflected at the metabolome level.

**5. Conclusion**

Although individual follow-up should be conducted to be completely certain of the caste history, thanks to our protocol, we obtained old nest-workers that were very unlikely to have been foragers before. The observed differences in proteome or metabolome therefore correlated strongly with their current age or caste. We observed that some functions were dependent on both age and caste of the individual (*e.g.* immunity), while others were only dependent on age (*e.g.* xenobiotics degradation). Only the digestive function seemed to be purely caste dependent. Studying the same samples in parallel at two omics levels allowed us both to confirm the results obtained with each approach, but also not to miss biological processes that might be less visible with either method. As the relative importance of caste and age may depend on biological processes studied, it is understandable that studies find conflicting results about the role of age and caste (Lucas *et al.* 2017b; Kohlmeier *et al.* 2018a). Two other castes were not considered in this study: males and queens. In most ant species, males live only during the short breeding period, so it would be difficult to study the influence of age on their physiology. But queens live up to ten times longer than workers (Keller and Genoud 1997). Thus, although it would take several years, a regular monitoring of queens during the course of their lifetime should provide valuable information on the mechanisms slowing down the ageing process in the long-lived reproductive caste of eusocial insects.

**Data availability:** Until the article is published, dataset and other electronic supplementary materials are available at https://ncloud2.zaclys.com/index.php/s/nLAdQ8Qrm43aAfi > Chapter 6

**Author contribution:** DH, FB, FC, CS and MQ designed the experimental protocol, MQ performed the behavioural observations and prepared samples before use in omics; CB and FB performed the proteomics workflow; DH prepared samples for metabolomics and performed the raw data processing; CV performed the LC-HRMS injections for metabolomics; MQ performed the whole statistical analysis and look for manual functional annotation of metabolites; FB retrieved KEGG functional information for proteins and metabolites; MQ wrote the first draft; CB, FB, CV and DH wrote methodological parts of this draft; all authors edited the first draft.

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# Conclusion | Part 3

## Complementary omics studies revealed distinct profiles according to caste and age

This third part brings together three innovative studies exploring the influence of a eusocial organisation on the physiology of the black garden ant. So far, no study had investigated the physiological differences between queens and workers, or between worker castes in social insects, through inferences based on proteome and metabolome analyses. **Table S1** summarises the findings of our three metabolomics and proteomics studies. Based on this table, we highlight the similarities and differences between our results, stressing the importance of combining omics approaches to conduct a full exploration of how sociality may covary with biological processes. The broader interpretations concerning ageing mechanisms and sociality are discussed in the overall conclusion part (next section).

*Distinct profiles of queens and workers are repeatedly confirmed.*

Chapters 4 and 5 show that workers express more proteins and metabolites linked to immunity or xenobiotics detoxification than queens. It suggests that the structure of ant colonies gives to workers the role of a shield against external threats, by limiting the contact between queens and pollutants or pathogens. These two environmental factors may be harmful by triggering oxidative stress, which is also found in the metabolomic and proteomic profiles of workers. Indeed, in chapters 5 and 6, workers and more particularly the foragers have metabolites reflecting oxidative damage. Finally, the omics profiles systematically found in nest-workers are linked to the nutrient absorption or digestive system.

*Omics methods are complementary to disentangle age from caste effects.*

Although several biological processes are confirmed by both proteomics and metabolomics (*e.g.* fewer pro-immunity molecules in queens, more active lipid metabolism in young workers), these two omics methods have their specificities (discussed in Chapter 6). Combining those methods may prove to be crucial as illustrated by converging detections of identical mechanisms at the metabolic or proteic levels. The complementarity of the two methods is also reflected by the heat maps in Chapter 6, where the metabolome seemed to be more associated with caste and to a lesser extent with age, but the proteome was influenced almost exclusively by age. However, non-converging detections may also help to better discriminate how physiology is modified with ant castes or age. In our studies, only proteomics highlighted an active reproductive function and an enhanced somatic maintenance in queens, as well as the overexpression of two cytoskeleton-related proteins in old workers. On the other hand, only metabolomics highlighted the presence of anti-cancerous molecules in female workers, the expression of oxidative stress (especially in old foragers), and of nutrient-sensitive pathways linked to longevity (autophagy, FOXO, sirtuins). Besides, metabolomics gives an idea of the class of molecules that most

**Table S1: comparative table of our major findings by omics methods.** The first column gives the groups compared, the next ones indicate the chapter concerned and if it was a proteomics or metabolomics study. Unless otherwise specified, the presence of a biological process in a group means an overexpression of it compared to the other group (pairwise comparisons). To ease the inter-studies comparison, identical biological functions have a dedicated colour and are aligned when possible. The last line sums up general remarks from the studies not directly linked to group comparison. Concerning the age and caste effects, we use the same abbreviations as in Chapter 6: Y.NW = young nest-workers, O.NW = old nest-workers, Y.F = young foragers, O.F = old foragers.

| | Chapter 4 | Chapter 5 | Chapter 6 | |
|---|---|---|---|---|
| **Queens & workers** | **Proteomics only** | **Metabolomics only** | | |
| Queens | reproduction<br>somatic maintenance | | | |
| Workers | immunity<br>chemical communication<br>energy metabolism | immunity<br>xenobiotics detoxification<br>chemical communication<br>nutrient sensitive pathway linked to a decrease in life span<br>oxidative stress | | |
| **Workers only** | **Proteomics only** | **Metabolomics only** | | |
| Foragers | xenobiotics detoxification | immunity<br>saturated fatty acids<br>juvenile hormone precursors | | |
| Nest-workers | digestion | digestion<br>nutrient sensitive pathway linked to an extended life span | | |
| **Age effect** | | | **Proteomics** | **Metabolomics** |
| *Y.NW vs O.NW* | | | lipid metabolism<br>xenobiotics detoxification<br>cytoskeleton | lipid metabolism<br>anti-cancer |
| *Y.F vs O.F* | | | immunity<br>more lipid metabolism<br>less carbohydrate metabolism<br>xenobiotics detoxification<br>cytoskeleton | immunity<br>chemical communication<br>less oleic acid derivatives<br>more lipid metabolism<br>less carbohydrate metabolism<br>xenobiotics detoxification<br>less oxidative stress<br>anti-cancer |
| **Caste effect** | | | **Proteomics** | **Metabolomics** |
| *Y.NW vs Y.F* | | | digestion | anti-cancer |
| *O.NW vs O.F* | | | digestion<br>immunity | immunity<br>chemical communication<br>less oleic acid derivatives<br>anti-cancer |
| **Remarks** | **Chapter 4** | **Chapter 5** | **Chapter 6** | |
| | Mid intra-group variacne<br>Failed to find mTOR pathway | Mid intra-group variance | Very low intra-group variance<br>Omics method are complementary.<br>Both age and caste impact the worker physiology. | |

differentiate the castes: in our case, the metabolism of lipids was strongly represented by molecules involved in immunity, cuticle composition, hormones synthesis, chemical communication, or energy reserves.

*Disentangling the age and caste effects on worker physiology helps to refine previous conclusions.*

Chapters 4 and 5 showed that workers had more metabolites and proteins associated with chemical communication than queens. But Chapter 6 made clear that old foragers did not overexpress them, apart from some oleic acid derivatives. Similarly, Chapter 4 showed that workers had a more active metabolism in general, but Chapter 6 highlighted an age-dependent preferential use: lipid or carbohydrate mobilization in the younger and older ants, respectively. As we have already seen, Chapters 4 and 5 showed that compared to queens, workers had a higher abundance of proteins and metabolites related to the immune system and detoxification of xenobiotics. However, while Chapter 6 also found those molecules in the two castes of workers, their expressions seemed to decrease with age. At the same time, oxidative stress was mainly observed in older workers. We hypothesised that the adaptive response against oxidative stress could lead to an energy trade-off, resulting in the lower quantities of molecules involved in immunity and detoxification of noxious xenobiotics observed in old foragers. Finally, the separation of the age and caste effects in Chapter 6 has enabled us to reduce the inter-individual variance among worker groups observed in Chapters 4 and 5. So, although in these first two chapters we had workers of homogeneous age, the control was not as strict as in the last chapter, which seems to have induced noise in both the ant metabolome and proteome. Thus, controlling for age in future studies appears to be essential if the effects of caste alone wish to be assessed.

*Study of ageing and selection of long-lived individuals.*

In Chapter 4, the ants studied were about 2 years old, which is beyond the average life expectancy found in this species (309.23 ± 36.24 days; Kramer *et al.* 2016). It is therefore possible that we may have unintentionally selected individuals that developed particular longevity mechanisms. This may explain why we did not find any proteins linked to signaling pathways involving sirtuins, mTOR, or FOXO, contrary to chapter 5. Although we found a greater quantity of proteins linked to somatic maintenance in queens, a possible selection of long-lived individuals could reduce the contrast between queens and workers. More broadly, in all studies analysing the effect of ageing through measurements in old individuals, the question might then arise to know whether such measurement reflects ageing mechanisms or mechanisms enabling those peculiar individuals to reach a later age.

# Overall conclusion

We saw in the introduction that trying to explain ageing and death is one of the oldest questions of humanity. Today, although we have left aside the promises of alchemy to conquer eternal life thanks to the philosopher's stone or fountain of youth, the quest to extend healthy lifespan has not ceased. However, this quest is no longer confined solely to the satisfaction of an individual desire. Indeed, human exploration of deep space involves manned spaceflight over a very long period of time. Thus, the question now arises at the level of humanity: will we be able to live long enough or slow down ageing sufficiently to allow manned space flight on a scale of decades or more (Criscuolo *et al.* 2020)? This thesis, of course, provides neither the recipe for eternal youth, nor the means to enter stasis and wake up a century later in perfect health. My research work does not even concern, at first sight, human health. However, even with human research in mind, studies of non-human models are of paramount importance. As Snyder-Mackler and collaborators (2020) point out, these studies make it possible (i) to reduce the number of confounding factors; to carry out (ii) studies over several generations much more quickly; and (iii) invasive experimental studies, impossible in humans. Thus, the conclusions reached in this work are of interest not only to the passionate entomologist or ornithologist but also to anyone interested in better understanding the mechanisms of ageing and more importantly how these can be linked to the social environment.

## Ageing modulation by the social environment: an effect reinforced at high levels of sociality

The four ant studies presented in this thesis describe the multiple effects of sociality on ageing parameters. First of all, **Chapters 4, 5 and 6** demonstrate that the caste profoundly and finely shapes both the proteome and the metabolome of ant queens, but also of the different worker castes, inducing important physiological differences (*e.g.* digestion, immunity, detoxification). The eusocial way of life would allow queens to make energy compromises in favour of her reproduction and longevity at the expense of other functions. Hence, longer lifespan may result from energy re-allocation. However, this energy trade-off is only possible because of the eusocial organisation of the colony, with the workers performing the digestive and immune functions instead of the queens. **Chapter 3** tested the effect of sociality on ageing more directly with a sociality index corresponding to species' levels of division of

labour. We were thus able to show that in the queens of the most social species the maximum life span potential (MLSP) was longer. On the contrary, sociality did not explain the MLSP of workers. This study therefore shows that it is the reproductive individuals that are most affected by the level of sociality regarding MLSP, particularly in species where the division of labour is the strongest. This is in line with the review by Keller and Lucas (Lucas and Keller 2020) which concluded that when comparing different species with different sociality levels, the benefits of sociality appear to be greatest for the reproductive individuals of highly social species.

Nevertheless, even if sociality may not influence the maximal longevity of less social species, it affects the ageing rate of individuals, even at low sociality levels. For example, **Chapter 1** shows that aggressive social interactions lead to a decrease in telomeres, notably via the oxidative response. The same chapter also suggests, even if it requires more statistical power to be fully conclusive, that the increase in aggression is due to a more crowded social environment. Finally, in **Chapter 2**, this social environment appears to be of prime importance in the early stages of development. Indeed, the presence of helpers in the first days after hatching leads to longer telomeres in sociable weavers. In the same study, longer telomeres in 9-day-old chicks were found to predict survival up to five years after fledging, thereby influencing life-history parameters related to individual fitness.

## Social insects, an exception to the classic mechanisms of ageing?

In the first two chapters of this thesis, as well as in many studies in birds or mammals, telomere shortening and oxidative stress were positively associated with ageing rates. Social insects generally appear as an exception to this global pattern. However, these conclusions have been drawn from only a few species, and their broader significance among the diversity of social insects should be addressed. We sought to find out whether social insects, here 10 species of ants, really exhibit an ageing phenotype that is not reflected by widely used markers of ageing: oxidative stress and telomere length. Regarding telomere length, it was independent of worker MLSP and even shown an opposite relationship in queens: the longer they live, the shorter the telomeres. Telomeres then do not apparently reflect the exceptional queen longevity, as other studies have shown previously (Jemielity *et al.* 2007; Korandová and Frydrychová 2016). On the other hand, the metabolomes of ants showed that the groups with a theoretical more aged phenotype (workers *vs.* queens, foragers *vs.* nest-workers, old *vs.* young) were those with more markers of oxidative stress and/or with a stronger response to it. Furthermore, the interspecific analysis pointed out that among queens with a high sociality index, those with the least DNA damage and with a greater antioxidant capacity were the ones who live the longest. In the light of these three studies, the ageing of ants therefore seems to be at least partly reflected by the oxidative

balance (not to mention a causal link, not tested here), as previously described in the honey bee (Haddad *et al.* 2007; Hsieh and Hsu 2011a, b; Tolfsen *et al.* 2011), but in a social-dependent way.

On the other hand, studies have found that neither antioxidant enzymes (SOD and catalase) nor glutathione were associated with greater insect longevity (Parker *et al.* 2004; Corona *et al.* 2005; Schneider *et al.* 2011). Our results (**Chapter 3**) confirmed the lack of influence of SOD on MLSP, but total (non-enzymatic) antioxidant capacity was associated positively with MLSP. I therefore hypothesise that the general mechanisms could be the same: ageing individuals accumulate oxidative damage. However, the target molecules could be different. Indeed, I realised during the DNA extraction step (see **Foreword** in C**hapter 3**) and the analyses of the metabolomics results, that ants have a body composition very rich in phenols and terpene derivatives, molecules usually present in plants. Similarly, several metabolites identified in ants had also been better described in plants than in other animals. It has also been shown that insects regenerate ascorbate enzymatically, just as green plants do, whereas vertebrates do not (Felton and Summers 1995). Finally, insects have very little circulating glucose, because the majority sugar of the haemolymph is trehalose ((Thompson 2003 p. 207); **Chapter 6,** in greater quantities in old than in young foragers). Trehalose has antioxidant capacities and could also be involved in the activation of autophagy independently of the mTOR pathway (Felton and Summers 1995; Mayer *et al.* 2016). Hence, these various isolated indices lead us to question the relevance of usual markers of oxidative stress in insects. By establishing new markers, more relevant to insect physiological particularities, we might more accurately test the hypothesis of oxidative balance as a biological marker of social insect ageing.

## How universally regulated is ageing?

Although telomere length appears to be a very good predictor of longevity in mammals and birds, we have seen that **Chapter 3**, like previous studies, suggests that this is not the case in social insects. However, comparative studies in small mammals have shown that telomere length do not always act as a marker of somatic ageing, and (together with telomerase activity) has negatively coevolved with body size thereby limiting the risk of cell immortalization with age in large animals (Seluanov *et al.* 2018). Having shorter telomeres and a telomerase shut-off trigger the replicative senescence earlier, which limit the number of possible divisions *per* cell and therefore the appearance of tumours. The theory here states that for a short-lived animal investing in anti-cancer mechanisms is not profitable, as it will have died before the serious after-effects of cancer appear. Conversely, a long-lived animal will be more likely to develop cancer and die of it before the age it could have reached by investing in anti-cancer mechanisms. Given the life expectancy of queens (up to 20 or 30 years), the probability of developing cancer during their long life is high. Accordingly, **Chapter 3** showed that queens living

longer had shorter telomeres. Besides, **Chapter 6** noted the presence of anti-cancer metabolites in all groups of workers. This might suggest that all castes, not just queens, may set-up anti-cancer mechanisms. Although *Lasius niger* workers live shorter than queens, they live longer than the adult forms (imago) of solitary insect: respectively 3 years (Kramer *et al.* 2016) and 0.1 ± 0.2 years (average lifespan among 87 solitary insect species from 8 orders; Keller and Genoud 1997). While tumours are found in insects (Tascedda and Ottaviani 2014; Aktipis *et al.* 2015), this putative resistance to cancer has yet to be demonstrated experimentally. However, such a demonstration has already been made in another eusocial species: the naked mole-rat (*Heterocephalus glaber*). This eusocial rodent can live up to 32 years and shows an incredible capacity of resistance to tumour development based on multiple protective mechanisms (Seluanov *et al.* 2018).

The second point of convergence between queen ants and more distant taxa is the effect of diapause on telomeres. **Chapter 3** showed that queens belonging to species with diapause had longer telomeres. A similar pattern has been found in hibernating mammals (e.g. Turbill *et al.* 2011). In addition to showing the generality of the consequences of diapause, it also underlines the importance of life-history traits in explaining the mechanisms of ageing.

Finally, **Chapter 5** highlighted nutrient-sensitive pathways linked to longevity (FOXO, sirtuins, regulation of autophagy or mitochondrial activity). Known for a long time in worms (Kimura 1997), such pathways have been extensively explored to understand the mechanisms of ageing in various taxa (Blagosklonny 2008). They establish a link between energy balance, activation of the metabolism and longevity. In our study, queens did not express EPEA, a metabolite linked to nutrient sensing and to decreased life span in *C. elegans* (Elphick 2012; Connor and Watts 2019), while nest-workers, compared to foragers, over-expressed nicotine amide and glutamate, respectively involved in sirtuins and FOXO pathways, both known to extend life span (Greer *et al.* 2007; Sedding 2008; Anderson and Prolla 2009; Morselli *et al.* 2010; Someya *et al.* 2010). Hence, nutrient-sensitive pathways do not only explain the gap in longevity between queens and workers, but also appear to underline the slighter difference in ageing rates between nest-worker (slower ageing rate) and foragers (faster ageing rate). However, the molecule we highlighted are upstream elements of those pathways and could also be involved in other biological processes. Targeted transcriptomics or proteomics should be carried out to determine precisely how these pathways are modulated by sociality in ants.

To conclude, we found shared regulatory mechanisms of ageing across taxa. Independently of phylogeny: (i) long-lived animals may depend on anti-cancer mechanisms; (ii) telomeres are lengthened during periods of low metabolic rate (diapause, hibernation); (iii) nutrient-sensitive pathways appear to be conserved key regulators of life span.

## Future challenges in the understanding of ageing mechanisms.

This thesis brings innovative aspects to the links between sociality and ageing at several levels. **Chapter 1**, studying zebra finches, established a causal chain between several physiological and behavioural responses, usually studied separately (aggressiveness, oxidative stress, telomere attrition, food preference). **Chapter 2**, contrary to most studies in cooperative breeders, focused on the effect of helpers not on the parents but on the offspring. Furthermore, it is one of the few studies to demonstrate in a wild population a positive link between telomere length very early after hatching and long-term survival. However, the parts that have provided both the most technical challenges and novelty are the studies of ageing mechanisms in social insects. We have performed the first multi-species study interested in oxidative stress and telomere length in ants and thus complemented the available data on this topic, which so far only concerned honey bees, bumblebees and black garden ants. However, we had to face many technical and methodological challenges: improving the DNA extraction protocols for qPCR, finding a control gene compatible with the 10 species in the study, designing a suitable primer for this control gene, adapting the PCR conditions. Finally, we have also developed a eusociality index as a variable that makes it possible to evaluate the influence of sociality on life expectancy, telomere length and oxidative status of queens and workers. In the last part, the innovations brought by the thesis consisted in four major points: i) to use proteomics and metabolomics rather than transcriptomics; ii) to combine and compare the results of these two methods; iii) to have not only studied the queen/worker but also the nest-worker/forager differences; iv) to have separated the effect of age and caste by another means than manipulations of the demographic structure (in order to have old nest-workers that have never been foragers). These pioneering studies on ants provided some answers, but also, as often in science, many new questions. Below, I address three points which, according to the findings of my thesis, seem central to a better understanding of the mechanisms of ageing in social insects: the impact of eusociality in the compromise of life-history traits, the ubiquity of telomerase, and experimental designs.

### *Impact of eusociality in life-history trait trade-offs*

One of the characteristics of eusociality is the reproductive division of labour: the queen monopolises sexual reproduction within the colony. As queens are also the longest living members of the colony, there seems to be a contradiction of the trade-off between fertility and longevity usually observed in non-eusocial species (Kirkwood *et al.* 1991). In one way or another, the eusocial lifestyle therefore gets queen ants rid of this energy compromise. Our studies have highlighted two biological processes in which queens could potentially invest less and reallocate the surplus both in their fertility and in maintenance mechanisms: immunity and digestion metabolism. The digestion metabolism seems

a particularly promising avenue to explore since pathways known to link longevity and energy balance (*e.g.* mTOR, sirtuins) were expressed differentially between ant castes. This would therefore be a key avenue to explore searching for the modulation of longevity by social role. Immunity also plays a key role in the evolution of eusocial insects. There is currently a consensus at the species level that selection for a eusocial lifestyle has been accompanied by a loss in the number of genes linked to immunity (Libbrecht *et al.* 2013; Roux *et al.* 2014; López-Uribe *et al.* 2016; He *et al.* 2020). At the individual level, Chapters 4 and 5, as well as some other studies (Judice *et al.* 2006; Lucas *et al.* 2017b) have shown that queens express less immune function than workers. However, several transcriptomic studies find the opposite relationship (Graeff *et al.* 2007; Grozinger *et al.* 2007; Lucas and Keller 2018). Many reasons discussed in Chapter 5 may explain these differences in results (*e.g.* different methods of analysis used, molecules that do not only have an immune function, age and caste effects). Providing a definite answer requires to actually test the pathogen resistance of ant castes. Chapter 6 and previous studies in termites and bees (Stucki *et al.* 2017; Li-Byarlay and Cleare 2020) showed that both age and caste can affect immunity. We therefore propose to use a protocol similar to the one used in that chapter to obtain colonies where all the ants are young (including the queen) and older colonies where all the ants are older (including the queen). All ants in a colony would be infected with a pathogen and the resistance of each individual would be analysed using several indicators: survival probability, haemocyte count, and measurement of phenoloxidase activity. This would provide an experimental cue of the effectiveness of the immune response depending on both age and caste. Varying the source of pathogens (bacteria, viruses, ectoparasites, and fungi) might highlight a different immune response depending on the types of pathogens that the castes are likely to encounter.

### *The ubiquity of telomerase in ageing mechanisms*

Telomere shortening at each division leads to the programmed death of cells at the end of a certain number of divisions (Shay and Wright 2000), and then to senescence of the organs and the whole organism. Being able to lengthen the telomeres with the help of telomerase would therefore make it possible to bypass replication senescence and extend life span. This is a strategy that seems to be adopted by certain organisms such as lobsters (Klapper *et al.* 1998) or long-lived birds (Haussmann *et al.* 2007). The rare studies in social insects have found that reproductive long-lived individuals had a higher telomerase activity than non-reproductive, shorter-lived workers in *Harpegnathos saltator* ants (Bonasio *et al.* 2010), bumblebees (Koubová *et al.* 2019), honey bees (Korandová and Frydrychová 2016); but only a slight, not significant difference between castes was found in the black garden ant (Jemielity *et al.* 2007) and the *Camponotus floridanus* ant (Bonasio *et al.* 2010). Besides, TA-65, a telomerase stimulating molecule, has shown its ability to extend the life span in adult laboratory mice (Jesus *et al.* 2011), improve human health (Harley *et al.* 2010), and help feather regeneration in birds (Reichert *et al.*

2014a). On the other hand, immortalizing cells by suppressing the control of replicative senescence and overexpressing telomerase strongly favours the appearance of cancers (Harley *et al.* 1994; Shay and Wright 2011). This is why large-sized, long-lived mammals are believed to inhibit telomerase coupled with other anti-cancer molecular pathways (Tian *et al.* 2018; Seluanov *et al.* 2018). In Chapter 3 we also showed that diapause had a beneficial effect on telomere length. In hibernating bats (Wang *et al.* 2011) and pre-diapause bumblebee queens (Koubová *et al.* 2019), increased telomerase activity has also been observed. Finally, accumulating data suggest that telomerase does not only participate in telomere lengthening but also fulfils other functions, called the non-canonical functions of telomerase. For instance, telomerase has been proved to inhibit apoptosis (Xiang *et al.* 2000) but activate cell proliferation (Smith *et al.* 2003; Mukherjee *et al.* 2011), protect mitochondria under oxidative stress (Ahmed *et al.* 2008; Haendeler *et al.* 2009; Saretzki 2009). Present in very distinct taxa and involved in various ageing-related mechanisms (*e.g.* anti-cancer processes, protection against oxidative stress, cell proliferation, telomere lengthening), the telomerase has the potential to be a common denominator to explain ageing mechanisms across the animal kingdom.

*Longitudinal studies and follow-up of wild animals*

As ageing concerns the evolution of an individual's physiology over time, longitudinal analyses appear to be essential in the study of such mechanisms. However, the size of social insects generally requires the experimenter to sacrifice the animal, which only allows a single sampling and therefore precludes longitudinal studies. The recent development of positron emission tomography (aka. PET scan) could allow us to imagine longitudinal protocols in social insects. The principle consists in designing a probe that will bind specifically to a particular cell structure by affinity (*e.g.* homologous sequence for DNA, ligand-receptor linkage for enzymes). This probe is radioactively labelled and injected, usually orally. The image is obtained by detection of photons when two positrons from the radioactive tracer annihilate each other. As the quantity of photons emitted is proportional to the amount of probe bound to the target, this technology makes possible quantitative measurement of biological processes (Peterson *et al.* 2008; Shoghi 2009; Willowson *et al.* 2012; Ahmadian *et al.* 2014). PET scan has already been used to study telomerase activity *in vivo* in mice (Groot-Wassink *et al.* 2004; Jung *et al.* 2016). It would therefore be possible, via food, to mark an entire ant colony. The ants could then be cold-anesthetised and the radioactive signal analysed. However, the smallest animals analysed so far with such a technology are mice, the size of which has nothing to do with that of an ant. Such system miniaturization would demand to solve technical issues such as injection volume, system sensitivity, spatial resolution (Yao *et al.* 2012).

Finally, I wanted to draw the reader's attention to the importance of confronting the hypotheses with the biologic reality of wild populations, with multiple factors interacting and modulating what can be observed in the laboratory. With the exception of the study on sociable weavers, the experiments carried out during my thesis were under controlled laboratory conditions. Likewise, throughout this manuscript, several laboratory experiments have been suggested to further our understanding of the ageing mechanisms. Such studies ensure that only the desired factors vary between the individuals studied. They are crucial in the first instance, reductionist approach, to test the different parameters one by one. Nevertheless, rearing conditions often deviate from natural conditions: *e.g.* less contact with pathogens, little or no competition for food resources distributed *ad libitum,* constant abiotic factors. Zajitschek et al. (2020) argue that these conditions may lead to erroneous conclusions, especially in ageing biology regarding the trade-offs between growth and senescence or the influence of feed restriction on longevity. This is the reason why the ultimate step to experimentally validate a hypothesis is to test it in the field. Is the hypothesis validated under natural conditions? If not, then the answer must still be incomplete and further work is needed to find the right hypothesis. If, one day, by dint of trials, rigour, but also probably with a pinch of luck, the right hypothesis is found. If this day Humanity unravels the mysteries of ageing and succeed in conquering immortality, then perhaps, before drinking at the fountain of youth, we should remember the words of writers and wise philosophers who have warned us about the dangers of immortality:

Quel est celui qui n'a pas rêvé à l'immortalité ? N'empêche qu'à un moment donné, ça doit commencer à poser de sérieux problèmes de bougies d'anniversaire…"

Who has never dreamed of immortality? Still, from a certain point on, it must cause serious birthday candle problems…"

Philippe Geluck / Ma langue au chat

# Appendices

# Appendix 1 | Additional article: network age, a new dimension in healthy ageing.

Cédric Sueur[1,2], Martin Quque[1,3], Alexandre Naud[1,4], Audrey Bergouignan[1,5], François Criscuolo[1]

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*Abstract*

While growing from children to adults and seniors, social relationships change. Social capital has been recognised as a strong mediator between age and health in animals and humans. Social capital changes with age, and influences the evolution of cognitive function, reproductive success, and mortality rate. This suggests that social capital is a new independent dimension standing in between ageing *per se* (chronological age) and senescence (biological age). It has been recently named the "network age". Here, we propose that network age is a modifiable variable that could be used as a novel tool to better understand the evolution of animal life history traits, the individual differences in lifespan, and thereby provide with new pathways of promoting healthy ageing.

**Keywords**

Social network; ageing; senescence; health; coevolution; social capital

*Introduction*

The 2020 Covid-19 outbreak is making us realise that a lack of social contacts can seriously harm our health (Armitage and Nellums 2020). Observations on the influence of social relationships on human health are not new, but animal studies are booming on this topic (Silk *et al.* 2003; Yang *et al.* 2016). In humans, a large body of research has shown that a lack of positive social relationships is a risk factor for all-cause mortality (Holt-Lunstad *et al.* 2010). Strikingly, the effect sizes are comparable to those of deaths attributed to smoking or obesity (Christakis and Fowler 2007, 2008). In animals, social isolation may conduct to serious damaged health (Aydinonat *et al.* 2014; Koto *et al.* 2015; Dawson *et al.* 2018). There is also strong and repeated evidence linking social relationships to various disease-related outcomes, but the mechanisms that explain these associations remain largely unknown. A series of complex and intertwined behavioural, psychological and biological pathways are likely involved (Berkman *et al.* 2014) (box 1).

These complex relations between social relationships and health persist in later life (Rook 2015; Brent *et al.* 2015), suggesting that the quality of these relationships can influence the individual rate of ageing and healthy ageing. Indeed, individuals, even from the same population, show great variability in their rate of ageing. "Ageing differently" means that at the same chronological age (*e.g.* 81 years old for a human), one may not have the same health status and/or mortality risk than another individual. The progressive deterioration of biological functions leading eventually to death is called senescence (physiological and cell mechanisms of senescence are developed in Box 1, as well as the link with the individual and network levels). While being a challenge, inter-individual variability in senescence also offers a unique opportunity to uncover the genetic and environmental factors that govern these differences among the members of a given population.

Individual social capital (Jenkins 2003; Silk *et al.* 2009; Brent *et al.* 2011), which is the structural (*e.g.* number of peers, time of interactions, relational patterns) and functional (*e.g.* social support, companionship) aspects of social relationships, can be influenced by senescence (Yang *et al.* 2016). As chronological age is positively related to deteriorated health at older ages (Pyrkov and Fedichev 2019), this may have reduced ability to maintain or create social relationships, so influences social capital (Nicholson 2012). A similar process is observed in animals with social interactions rate decreasing when the rate of activity decreases (Fischer 2017; De Waal 2019). From an individual perspective, social capital is constantly evolving in response to life events and thus changes with chronological age (Wrzus *et al.* 2013).

The link between social capital and survival has been observed in a number of vertebrates both humans and non-humans (see Snyder-Mackler *et al.* 2020 for a review). Physiological changes in ageing are subject to natural selection and social animals share evolutionary constraints and selection pressures that may result in common physiological changes with age. Although the interspecific association between social capital and longevity has been largely investigated, the dynamic of the covariation between social capital and senescence has remained overlooked. We subscribe to the view that integrating the study of the dynamic interactions between social capital and ageing could provide new and innovative perspectives to research on healthy ageing and longevity.

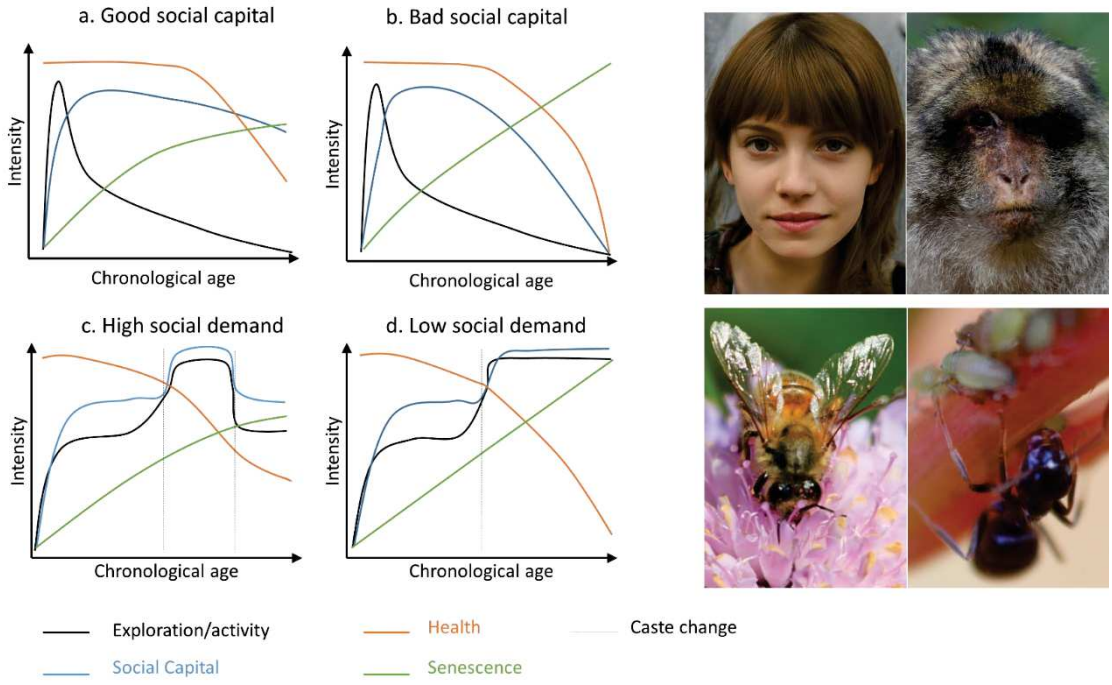



**Figure 1: Evolution of senescence and health** for a good **(a.)** or a bad **(b.)** social capital in humans and macaques and for a low **(c.)** and a high **(d.)** social demand in eusocial animals (e.g. bees and ants). These species have comparable age trajectories making ants, bees, macaques, humans and surely other social animals as elephants or killer whales, good subjects to study healthy ageing. Curves are theoretical and based on research conducted in each species and cited in the main text. They represent the global trajectory of the dimensions over the lifetime of each species. Health is a state of physical, mental and social well-being depending on internal factors (senescence) and external ones (pathogens, pollutants, etc.). When health goes to zero, individuals die. Bee, ant and macaque pictures are from Cédric Sueur. The human picture was generated by StyleGAN, a generative adversarial network (GAN). The person in this photo does not exist but is generated by artificial intelligence based on an analysis of portraits. This human picture is in the public domain because, as the work of a computer algorithm or artificial intelligence, it has no human author in whom copyright is vested

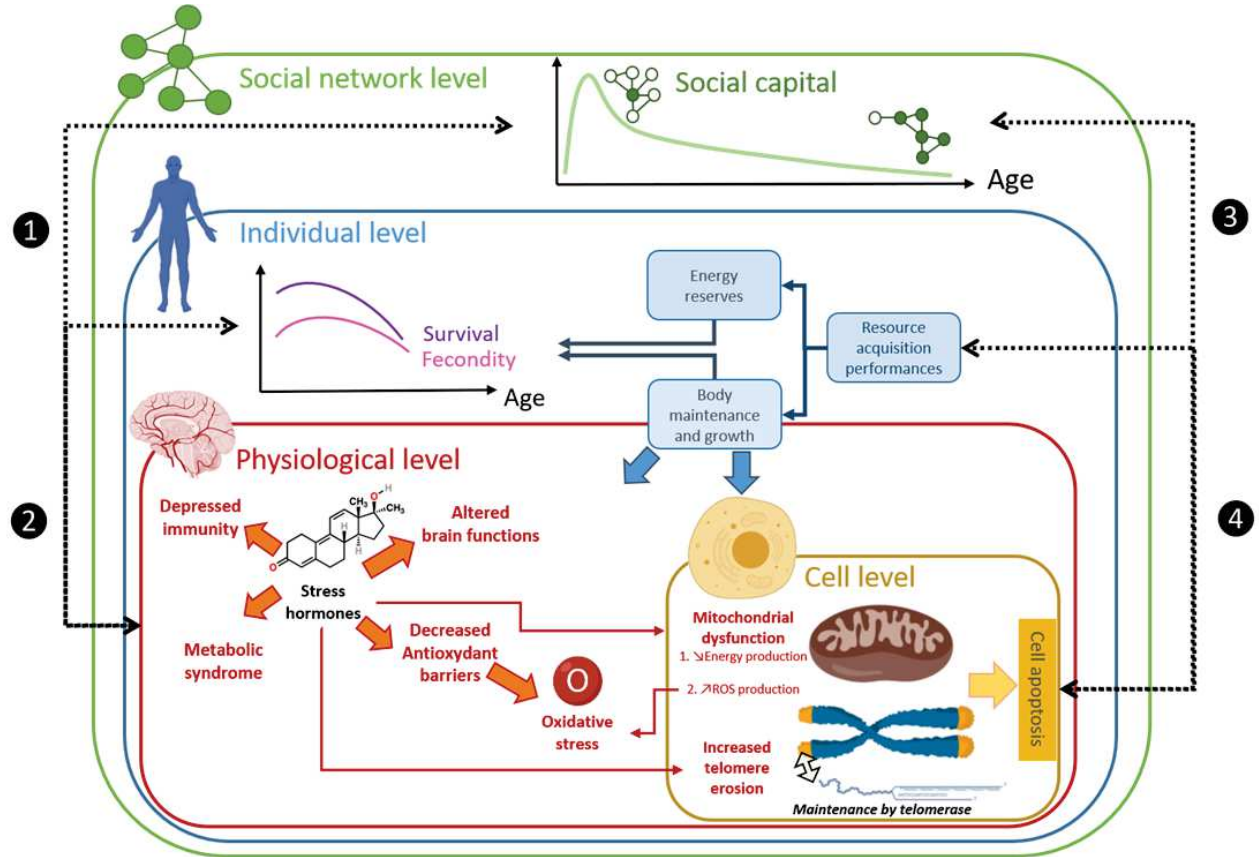
*Impact of chronological age on social capital*

Social capital evolves with age and a general pattern has been identified and described. In humans, children focus on few but strong relationships with individuals who share similar characteristics (*e.g.* gender, relatives, hobbies). These few relationships are the basis to learn rules of life and sociality.

Similar observations have been reported in non-human animals (Shimada and Sueur 2014, 2018). Then, adolescents and adults expand the quantity and diversity of their social relationships (Freund and Baltes 2000), and as they get older they become more selective (Field and Minkler 1988) (figure 1). Two prevalent theories, namely the socio-emotional selectivity and social convoy theories, propose an explanation for the lifespan development of social relationships. If these theories predict similar changes, they attribute these changes to different causes (Wrzus *et al.* 2013). According to the socio-emotional selectivity theory (Carstensen 2006), young people are more likely to feel that time is open-ended, and therefore focus on goals related to knowledge acquisition and select relationships accordingly. By contrast, seniors are more likely to feel that time is limited. Consequently, they rather focus on increasing their well-being, and on a social life organised around a few deeply satisfying relationships. Similar results were found in macaques (Almeling *et al.* 2016; Crockford 2016) and elephants (Archie *et al.* 2006). However, non-human animals most likely have no conception of their limited lifetime. Comparison between human and non-human animals may therefore allow disentangling the effects of cognitive insights from physiological processes that may contribute to changes in social relationships. In the social convoy theory, individuals go through life while being embedded in a constant personal network of individuals from whom they give and receive social support. In Westernised societies the value of seniors is considered minimal by younger generations. The social convoy of seniors is therefore often limited to their spouse and close family members, thus isolating them from the rest of the society. However, Almeiling et al. (2016) reported that old macaques seem to remain valuable to young macaques who keep grooming them for social cohesion and tolerance. These authors proposed that the maintenance of social relationships with older individuals may contribute, at least at the group level, to promote health status and lifespan. These health benefits are likely the result of alliances, knowledge and/or well-being through endorphin secretion in animals from all age (McComb *et al.* 2001; Almeling *et al.* 2016). In animal societies, knowledge is a key resource provided by older group members. As individuals age, their role in the group and hence their social capital evolves because of their expertise and leadership (McComb *et al.* 2001; Tokuyama and Furuichi 2017; Nattrass *et al.* 2019). A parallel on the importance of social role linked with age could be drawn with eusocial insects such as ants or bees, in which non-reproductive workers change of social role (*i.e.* caste) according to their age (*i.e.* temporal polyethism). Throughout ontogenesis, worker ants or bees change of castes in an age-dependent manner (Münch *et al.* 2008). Young workers typically perform within-nest activities like nursing larvae, and after some weeks, they switch to foraging duties collecting foods in the environment for the colony. Interestingly, these insects show some age-related cognitive decline (Baker *et al.* 2012) and changes in their social interactions (Mersch *et al.* 2013); the latter being directly dependent on their role in the colony. Bees and ants are also able to reverse their castes (from

foragers to nest workers) and modify their interactions if a demand appears in the colony (*e.g.* following a nest predation event). This social reverse affects the senescence status of the individuals showing an interplay between these two variables (Amdam *et al.* 2005; Baker *et al.* 2012). The reverse in their social role can reverse their senescence.

**Box 1: Biology of ageing, senescence, and longevity in social animals**




For a decade, social stress has been pointed out as a modulating factor of ageing pathways (Blackburn and Epel 2012), with potential therapeutic possibilities (Epel *et al.* 2004). Still, the change in social capital over age (network age) underlines putative loop of intertwined pathways to explore. ❶ Social environment affects individual fitness (survival and fecundity) *via* direct effects on survival or reproductive success via phenotypic changes and even resilience of the future individual to social stress. ❷ Social isolation and interactions have been suggested to have opposite effects on stress hormones (Wittig *et al.* 2016) with potentially negative consequences but also adaptive responses at the physiological and cellular level (*e.g.* oxidative stress) (Katyare *et al.* 2003). Among those, increased telomerase activity in socially stressed individuals was previously described (Beery *et al.* 2012). In bees, the age-dependent cognitive decline can be reversed by switching caste (Baker *et al.* 2012). ❸ Social isolation was suggested to have pervasive effects on subsequent performance (Ballen *et al.* 2014). These altered individual performances in energy acquisition from the environment will be reflected in the life-history trade-offs on energy allocation to traits defining individual fitness. ❹ Social isolation triggers an increased rate of telomere loss (a biological index of ageing) (Aydinonat *et al.* 2014) and disrupts energy homeostasis. This suggests that social variables may influence cell ageing proxies, as previously suggested for social rank and telomere length. *Images are from Biorender*.

*Interplay between social capital, biological age and senescence*

Chronological age affects social capital, which in turn affects biological age. However, this bidirectional relation is likely to be dynamic and to vary according to a number of individual and contextual factors. These factors can include prestige, family, place of residence in humans, and

dominance, size of kinship, caste, habitat in non-human animals. Variations in biological age may affect social capital and performance in resources acquisition and ultimately fitness (see box 1). With age, individuals are more selective, and their interactions are restricted. The social environment influence is therefore stronger and can be either positive or negative. For example, it can lead to a potential increase in the risk of morbidity if a person's social relationships include a large number of friends who adopt risk-prone behaviours, like smoking or associated to obesity (Christakis and Fowler 2007, 2008). On the contrary, an active and socially integrated lifestyle in late life might protect against physical and cognitive decline (Fratiglioni *et al.* 2000). In non-human animals, a good social capital is not only linked to a better fitness in terms of protection against competitors or predation but also in terms of social grooming, learning about food resources, tool use, self-medication or even cultural evolution (Duboscq *et al.* 2016; Street *et al.* 2017). Social capital also impacts physiological functions and ageing markers. In humans, social isolation negatively modulates cognitive skills at old age (Holt-Lunstad *et al.* 2010; Nicholson 2012). Conversely, engagement in social activities is beneficial (Fratiglioni *et al.* 2000). In mice, deficits in social behaviour precede cognitive decline (Boyer *et al.* 2019). Social isolation causes mortality by disrupting energy homeostasis in ants (Koto *et al.* 2015). Because of their hyperactive behavioural pattern, single ants faced an acute increased energy demand and finally died. In fruit flies, cancerous flies are socially avoided by conspecifics; this social isolation has been shown to significantly accelerate the progression of tumour growth and death (Dawson *et al.* 2018). In a nutshell, health affects social capital, which affects in return health. In macaques, even if old members are more socially selective and groom fewer partners, they continue to observe the social life of other group members. It has been suggested that such social aspects stimulate brain activity, making social interest a key factor for at least cognitive ageing and most likely other aspects of healthy ageing (Fratiglioni *et al.* 2000). Remarkably, lifespan of eusocial insect workers ranges from a few weeks to more than one or two years. This plasticity is largely controlled by environmental factors (Münch *et al.* 2008), among which social environment is likely to be important. Thus, although individuals are closely related genetically, distinct life histories can emerge as a result of variations in their social environment. Researchers experimentally reversed the social role of old insect workers by decreasing the ratio of within-nest workers in ants and honeybees (Sendova-Franks and Franks 1993; Amdam *et al.* 2005). The sole change in social interactions resulted in molecular (Quque *et al.* 2019) and neuronal modifications (Münch *et al.* 2008) associated with reversible age-related learning deficits (Baker *et al.* 2012) (box 1). This result suggests that biological age is related to social network. A recent study conducted in bees (Wild *et al.* 2020) confirms that social interactions indices are better than chronological age at predicting task allocation, activity patterns and survival. A high social demand solicits more social interactions for workers and makes them have a

faster network change speeding up senescence and decreasing longevity. The authors named this new dimension "network age" (Wild *et al.* 2020).

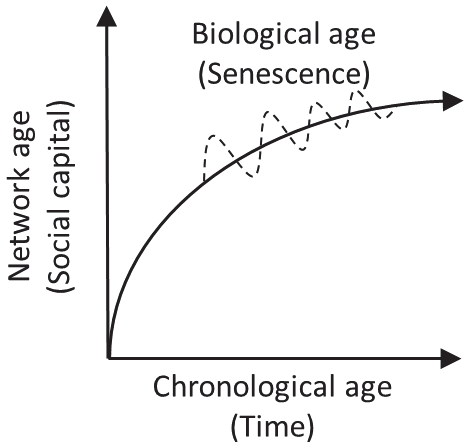



**Figure 2: Evolution of biological age according to chronological age and network age.** The dotted line represents variations observed in reversing caste and solicitations in eusocial insects but may result from intervention on social parameters in humans and other animals. Last research in animal species showed that biological age ③ is not only dependent on chronological age ① but also on network age ② with an interplay between ② and ③. If one should resume this in equations, this would give ③=α②*β①+γ and ②=δ③*ε①+θ with α, β, δ, ε being some constants depending on the impact of each variable for each individual/species; γ and θ being some constants dependent on other external factors and with α< β and δ< ε as chronological age cannot be inversed or slowdown.

*Perspectives: how to use animal model to characterise the role of network age in healthy ageing*

Biological age is only partly related to chronological age. This is likely because history in terms of stress or well-being is a key modulator of the rate of ageing (Blackburn and Epel 2012). As a consequence, biological age depends on different socio-environmental factors, including social network or what Wild and colleagues (Wild *et al.* 2020) named the network age (figure 2). Network age is characterised by the temporal changes in social capital. In macaques (Almeling *et al.* 2016) or elephants (Archie *et al.* 2006), changes in social capital follow the same patterns as the ones observed in humans, and these changes are linked to individual health and longevity (Snyder-Mackler *et al.* 2020). In bees, network age is better to predict task allocation and mortality than chronological age (Wild *et al.* 2020). The term "network age" seems to be adequate and useful to grasp specific aspects of ageing. The relationship between network age and biological age is likely to be bidirectional (figure 2 illustrates in equation this interplay).

Based on the current data obtained in both social animals and humans, we suggest that studying the influence of network age on biological age will bring novel and important information that will help advance the science of ageing: 1) for humans but also for animals, and 2) in an applied perspective but also a more fundamental, evolutionary one. To do so, we believe the following specific questions need to be addressed:

**1.** What are the components of network age in social animals? Most of the past studies in social gerontology as well as in animal social network science have only focused on the direct social connections between the elements of a network while indirect connections (*e.g.* friend of our friend) also seem to be important for social capital (Christakis and Fowler 2007, 2008). These indirect connections affecting network indices as betweenness (Sosa *et al.* 2020) may strongly affect the evolution of species

in which sharing of knowledge is important (Henrich 2017; Sueur *et al.* 2019). As a consequence, it could also influence their life history traits (Bribiescas 2020). Thus, there is a need to assess the different dimensions of network age by studying a larger spectrum of social indices. Obviously, components of network age in humans are likely different from those observed in bees or macaques. Future research needs to identify specific social indices in each species (*i.e.* the impact of direct *vs.* indirect connections, the influence of the quantity as well as the quality of relationships, etc.) in order to help understanding the relationship between network age and healthy ageing.

**2.** How does network age evolve with chronological age and senescence? Investigating the interplay between social capital and ageing, and between social capital and senescence would stimulate multidisciplinary and integrative approaches going from physiology to sociology. Animal species characterised by specific network age can emerge as novel behavioural models to address relevant questions in current ageing research (*e.g.* the age-related change in fertility, (Monaghan *et al.* 2008)) and help to determine how network age is associated with life periods (*i.e.* early-life, reproductive life, post-reproductive life). For instance, an extended post-reproductive life may have been co-selected with specific social traits, due to their positive effects on individual fitness (Kirkwood 1977; Lahdenperä *et al.* 2004). In killer whales, females live twice longer than males and post-reproductively aged females have greater knowledge and lead the clan, enhancing the survival of their grand-offspring (Nattrass *et al.* 2019). What network age adaptations may have co-evolved with this sex-difference in longevity? This grandmother hypothesis also concerns humans (Hawkes *et al.* 1998; Lahdenperä *et al.* 2004). Taking into account network age will enable us to go deeper in our understanding of how changing our social environments can be used to improve quality of social life especially in later age.

**3.** How does network age influence biological age? The predictions of evolutionary theories of ageing could be tested in animals by focusing on network age in order to better understand its links with biological age and fitness (Reznick *et al.* 2005). For example, the Free Radical Ageing theory (Beckman and Ames 1998) and its caveats would have to be tested in the light of the network age theory. In the same context, the effects of social interactions on other key mechanisms of ageing, including mitochondrial dysfunction, metabolic syndrome, autoimmunity diseases and others, need to be evaluated because they may offer strong advances in evolutionary biology and applicable therapeutic solutions for healthy ageing. The use of omics (*e.g.* genomics, proteomics and metabolomics (Münch *et al.* 2008; Quque *et al.* 2019) in an evolutionary perspective will help to uncover how in bees or ants the molecular mechanisms underlying the relationship of social capital to health may have been co-selected. By asking the question of the coevolution of anti-ageing mechanisms and social capital, we propose a

novel vision to approach ageing. This implies to set up experimental designs to directly compare individuals of same chronological age but contrasted senescence status or social capital.

Most ageing and social studies, on humans or non-human animals, have been conducted using chronological ageing or social network as the key referential. However, to our point of view, this parameter only partly reflects the health status of an individual. Our traditional approach may have reached its limits and new paradigms are needed to achieve breakthroughs. Comparing health of individuals of same or different network and biological ages will prove to be more powerful than standing with chronological age. Of course, we cannot affect chronological age but studies presented in this paper showed that a reverse in network age can slowdown senescence. This new concept could bring new elements about the species diversity of lifespan and sociality. Time is finite for most living animals, but sociality seems to be a promising tool to make senescence adjustable.

# Appendix 2 | Additional article: Hierarchical networks of food exchange in the black garden ant *Lasius niger*

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[*] Equal contribution of these authors

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*Abstract*

In most eusocial insects, the division of labour results in relatively few individuals foraging for the entire colony. Thus, the survival of the colony depends on its efficiency in meeting the nutritional needs of all its members. Here, we characterise the network topology of a eusocial insect to understand the role and centrality of each caste in this network during the process of food dissemination. We constructed trophallaxis networks from 34 food-exchange experiments in black garden ants (*Lasius niger*). We tested the influence of brood and colony size on (i) global indices at the network level (*i.e.* efficiency, resilience, centralisation, and modularity) and (ii) individual values (*i.e.* degree, strength, betweenness and the clustering coefficient). Network resilience, the ratio between global efficiency and centralisation, was stable with colony size but increased in the presence of broods, presumably in response to the nutritional needs of larvae. Individual metrics highlighted the major role of foragers in food dissemination. In addition, a hierarchical clustering analysis suggested that some domestics acted as intermediaries between foragers and other domestics. Networks appeared to be hierarchical rather than random or centralised exclusively around foragers. Finally, our results suggested that networks emerging from social insect interactions can improve group performance and thus colony fitness.

**Keywords:** Social network analyses, Social evolution, Self-organisation, Insects, Network evolution

*Introduction*

There are many well-known advantages to living in societies: protection against predators, more efficient discovery of food sources and better access to information (Krause and Ruxton 2002). Another benefit of group living is food sharing (Isaac 1978; Kaplan *et al.* 1985; De Waal 1989; Stevens and Gilby 2004). Food sharing can occur in both social and non-social species, and its evolutionary origins have been studied using the predictions of reciprocal altruism (Trivers 1971), biological markets (Noë and Hammerstein 1995) and multilevel selection (Traulsen and Nowak 2006).

Food exchange is central to many animal societies, including eusocial species such as ants, termites, bees and even naked mole rats (Anderson 1984; Jarvis *et al.* 1994; Wilson and Hölldobler 2005; Nowak *et al.* 2010). In eusocial species, only a restricted number of individuals forage and retrieve food for the rest of the colony. Castes can be distinguished by their behaviour and even by their proteome (Quque *et al.* 2019). Given that trophallaxis, the mouth-to-mouth transfer of food, is the mechanism of food exchange, the exchange of food facilitates social recognition via the exchange of informative colony-specific hydrocarbons (Boulay *et al.* 2000), information on the location of food resources (Gil and De Marco 2005; Frasnelli *et al.* 2012) and even immune-related molecules (Hamilton *et al.* 2010; LeBoeuf *et al.* 2016). In the black garden ant (*Lasius niger*), the only food exchanged—aphid honeydew (*Aphidoidea sp.*)—is stored exclusively in the crop of individuals (Buffin *et al.* 2009). The foragers give food to non-forager workers (*i.e.*, nest workers, hereafter called domestics), which may then transfer the food to other domestics, including caretakers of larvae. These chains of exchanges allow food to be disseminated throughout the nest, from the entrance to the deepest chambers (Wilson and Eisner 1957; Lee Cassill and Tschinkel 1999).

At the entrance of the nest, the interactions between forager ants are fundamental as they regulate the dynamics of food harvesting (Gordon 1996; Schafer *et al.* 2006; Pinter-Wollman *et al.* 2011; Pinter-Wollman 2015). However, domestics possess essential information on the colony's needs. Exchange between foragers and domestics is spatially confined within an area close to the entrance of the nest in both the wild (Tschinkel and Hanley 2017) and the laboratory (Mersch *et al.* 2013). For instance, in *Pogonomyrmex badius*, foragers represent less than 5% of ants inside the nest 20 cm from the entrance and are completely absent in regions 70 cm from the entrance (Tschinkel and Hanley 2017). This observation illustrates the spatial fidelity of castes and how this fidelity may affect network topology (Pinter-Wollman *et al.* 2011; Pinter-Wollman 2015) through differences in the connectivity between colony members (Jeanson 2012; Mersch *et al.* 2013).

To maximise fitness, the number of trophallaxes necessary to transfer food from foragers to the queen and larvae needs to be minimised to make the process of food exchange as fast as possible. Thus, efficient food exchange networks should be favoured by selection given the restricted roles of each caste

and their spatial distribution within the colony (Sueur *et al.* 2019). However, ants do not have a single, optimal social network topology (Camazine *et al.* 2003). Each colony member does not have to evaluate the needs of workers and the role of their activities in a task, as the trophallactic interactions spread relevant information through the entire colony (Grüter *et al.* 2006). Nevertheless, ants are known to have collective cognition (Couzin 2009) or swarm intelligence (Bonabeau *et al.* 1999). As a result, simple interaction rules can explain the construction of complex structures, such as the Towers of Hanoi (Reid *et al.* 2011) and how ants make bridges (Reid *et al.* 2015). Stroeymeyt and collaborators (2018) showed that the interactions of ants can be modified to mitigate the spread of disease, as the network centralities of ants are critically important for the transmission of infections (Romano *et al.* 2016). This behavioural plasticity permits the topology of the interaction network to be modified so that the network can become less efficient (for definitions of network efficiency, see Ek *et al.*, 2015; Pasquaretta *et al.*, 2014; Romano *et al.*, 2018) and more modular to prevent epidemics. This social immunity is well known in eusocial insects (Cremer *et al.* 2007; Cremer 2019; Liu *et al.* 2019; Małagocka *et al.* 2019) but has also been documented in other taxa, such as primates, where this phenomenon has been called the social bottleneck hypothesis (Nunn *et al.*, 2015; Romano *et al.*, 2020; Romano *et al.*, 2018).

Despite the central role that trophallactic interactions play in the regulation of food flow in eusocial species, the way that chains of demands are established and how they ultimately impact network topology have been largely unexplored. Furthermore, most previous studies on these subjects have not considered the individuality and identity of the trophallactic partners (Buffin *et al.* 2012). One of the first studies to analyse the entire trophallactic network demonstrated a spatial reorganisation of worker positions facing starvation that accelerated the recovery of food stocks (Sendova-Franks *et al.* 2010). Waters and Fewell (2012) identified individuals and antenna interactions in *Pogonomyrmex californicus* and concluded that the regulatory motif of interactions they observed supports the hypothesis that eusocial insects are shaped by selection for network patterns that integrate functionality at the group (*i.e.* colony) level rather than at the individual level. Lastly, Greenwald *et al.* (2015, 2018) assessed the role of foragers and non-foragers in the dissemination of food; however, these studies did not take into account the global state of the colony or conduct a thorough analysis of the network topology of trophallactic interactions.

Given that self-organisation results in the emergence of adapted complex systems (Camazine *et al.*, 2003; Fisher & Pruitt, 2019), we tested for the efficiency of trophallaxis networks in garden black ants. Specifically, we evaluated whether food was exchanged non-randomly (*e.g.* unpredictability in the direction and time of trophallaxes), expecting that the pattern of food dissemination maximises the speed with which food circulates through the colony. In addition, we conducted laboratory experiments in which we monitored the behaviour of foragers in colonies of varying sizes and with and without

broods. Overall, we tested for the effects of three main factors: brood presence, the forager/domestic ratio and behaviour (which were also response variables) and colony size. Each ant was followed during the entire test using QR codes (Garrido-Jurado *et al.* 2014; Stroeymeyt *et al.* 2018), and all trophallaxes were scored. We used social network analyses to study the efficiency of food dissemination. Combining experimentation with network analyses, especially in social insects, is a powerful tool for studying the evolution of complex systems (Charbonneau *et al.* 2013; Mersch 2016; Sueur and Mery 2017). We made three major alternative hypotheses concerning the network properties of trophallactic exchanges (fig. 1):

a. Interactions are random, and foragers have the same social centralities as domestics. Confirmation of this null hypothesis would suggest that trophallaxes do not play an important role in food exchange. We did not expect to find random networks.

b. Food exchange occurs exclusively between foragers and domestics. This hypothesis predicts that foragers should show higher centralities than domestics.

c. Foragers transfer food to domestics, but some intermediary domestics are involved in the chain of food dissemination. This hypothesis predicts that these intermediary domestics should show forager-like centralities. Thus, these intermediary individuals would provide the link between the source (foragers) and the final destination of food.

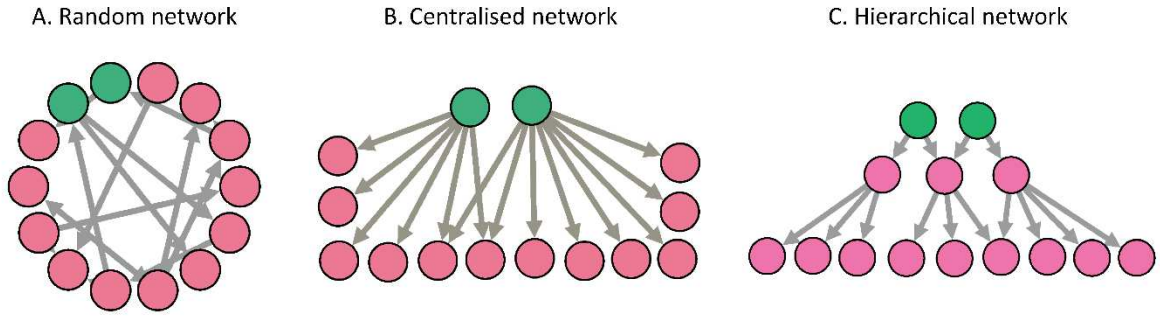


**Figure 1: Representation of the theoretical networks corresponding to each of the three hypotheses.** For each network, there are 14 individuals (two foragers and twelve domestics) as well as fourteen connections (*i.e.*, trophallaxes). a. Random network for which the trophallaxes are random between individuals, foragers or domestics. b. Centralised network for which trophallaxes only happen from foragers to domestics; there is one level of interaction. c. Hierarchical network for which trophallaxes are made first from foragers to domestics and then from these intermediary domestics to other domestics; there are at least two levels of interactions according to the colony size.

We used different social network indices to assess these predictions (see Table S1). We expected colony size to affect the network topology, as previous studies have shown that centralisation and modularity increases with colony size (Pasquaretta *et al.* 2014; Nunn *et al.* 2015). Efficiency should be stable because, theoretically speaking, efficiency should decrease with network size (Romano *et al.*,

2018); however, ants are capable of modifying their division of labour depending on the size of the colony (Jeanson *et al.* 2007; Holbrook *et al.* 2011; Modlmeier *et al.* 2019). Furthermore, the presence of broods is expected to modify the structure of the network, as broods affect both the nutritional needs of the colony and thus the demand for foragers (Portha *et al.* 2002). Because the presence of broods adds a level of food dissemination, brood presence should have an impact on both individual and global network indices with either the same ratio of foragers contributing more work (*e.g.*, via higher efficiency and higher centralisation) or more foragers decentralising the network.

*Material & Methods*

Ant colony setup

We created 52 queenless subcolonies of 11 to 120 workers, with and without broods (see details below) from 12 wild and large mother colonies (>1000 ants) of *Lasius niger* collected in Brussels, Belgium during the autumn of 2016. We formed the subcolonies after the colonies had been in the lab for 18 months years to decrease the potential effect of temporal polyethism. We tested queenless colonies for simplicity; several studies have shown that the absence of the queen does not affect the interactions between workers and the shape of the food dissemination network (Holbrook *et al.* 2011; Jeanson 2012; Bazazi *et al.* 2016; Bles *et al.* 2018).

These colonies were tested in an experimental enclosure (fig. 2a). The dimensions of the experimental enclosure were 17.5×12.5×5 cm. The walls of the foraging area were covered with Fluon®, in addition to a glass covering the tray, to prevent ants from escaping. Ants were placed in these trays for 10 days so that they could acclimate until the repartitioning of tasks among individuals stabilised. Although the division of labour is flexible according to the needs of the colony, several other studies have suggested that 10 days is enough for the caste of each ant to stabilise (Sendova-Franks and Franks 1993; Huang and Robinson 1996; Amdam *et al.* 2005; Baker *et al.* 2012). The colonies were kept at 22 ± 3 °C and 60 ± 5% relative humidity, with a constant 12:12 h photoperiod.

The experimental enclosure was divided into two parts: the nest area and the feeding area. Two food sources were placed in the feeding area at equal distances relative to the nest entrance: 0.3 mol/L of milk powder and 0.3 mol/L of sucrose. The position of the food sources relative to the nest entrances (*i.e.* left or right) was randomised. Ants were starved for 5 days in the nest area before experiments to increase foraging activity and the rate of trophallaxis. The experiments ran for one hour. Video data were recorded using a Panasonic® Lumix DMC-GH4-R mounted with a 30-mm Olympus® ED lens capturing 25 frames/s at a resolution of 4180×2160 p.

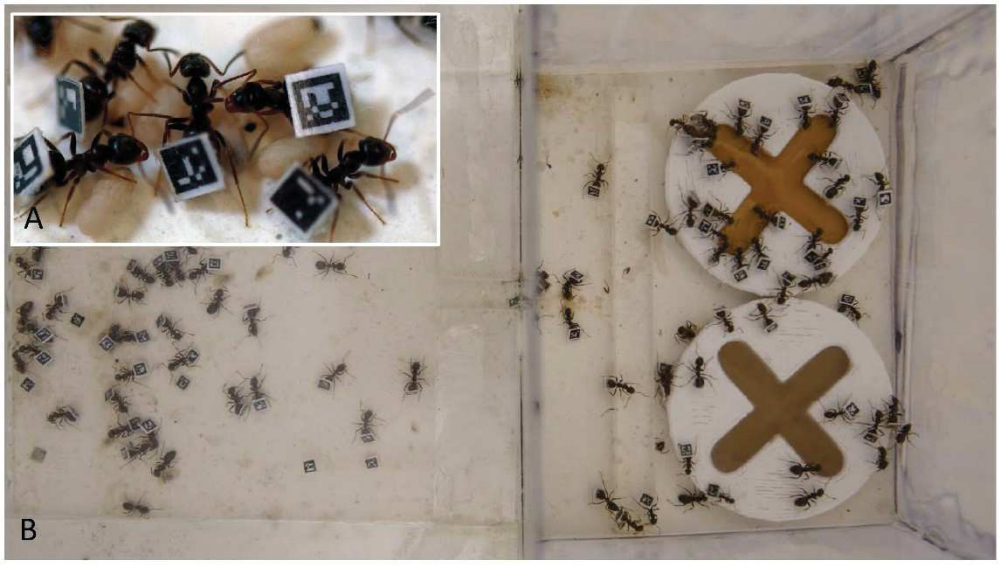


**Figure 2: (A)** Experimental setup for testing trophallaxes. Ants are placed in the nest area (on the left) where domestic stay whilst foragers go on the feeding area (on the right) where two food sources are placed: one with protein and one with sugar. The two food sources were randomly attributed. **(B)** Ants with Aruco tags (QR code) applied on their abdomen.

Ant Identification Through QR Codes

Labelling of ants with Aruco tags (QR codes, Garrido-Jurado *et al.* 2014), along with the software USETracker (https://sites.google.com/site/usetrackerac/), allowed individual ants to be identified continuously (fig. 2b). Ants were placed in the freezer until they were inert (about one minute). Each unique tag was then stuck to the abdomen and had a side length of 0.8 mm, weighed 0.1 mg (corresponding to approximately 5% of the average mass of an adult worker or less than 10% of the amount of food that a worker carries) and was printed on waterproof paper at a resolution of 1200 dpi. The tags were hand-cut using a scalpel and a steel ruler as a guide. Following a 5-min acclimatisation period, the labelling did not appear to impede the ant behaviours, movements, or interactions. We discriminated foragers from domestics. An individual was considered a forager if it spent at least 5 consecutive seconds feeding at the food source (not in the feeding area but at either one of the two food sources or both) during the experiment. This discrimination was possible by analysing the position of each individual per frame during the experiment (see fig. S1 in the supplementary material).

Data Scoring

Each trophallaxis that occurred in the entire observation area, its duration as well as the identities (unique tag) and caste (forager *vs.* domestic) of both the donor and the receiver ant were scored. A trophallactic event was recorded when ants engaged in mandible-to-mandible contact for greater than 2 seconds (the minimum time needed to exchange a piece of food). The directionality of the food flow and the role of the donor and the receiver were determined by body posture and mandible positions: The donor opens her mandibles and displays a droplet of sucrose solution between them while the receiver turns and moves her head forward to receive it (Greenwald *et al.*, 2015; Lee Cassill & Tschinkel, 1999).

We only analysed videos where 100% of the trophallaxis interactions could be identified. Of the 52 experimental runs, a total of 34 videos were analysed. Each of the 12 colonies was tested 2.8 ± 1.1 times (min = 1, max = 5). On the 34 videos, broods were present in 21 colonies and absent from 13 (proportion per colony: 0.66 ± 0.24). The colony size ranged from 11 to 120 ants (mean = 42 ± 26). Colony replications were incorporated into the statistical analyses. Different observers scored the videos; however, we found that there was weak inter-observer bias, as an inter-observer reliability test on eight videos revealed that the minimum score that we observed was 88.5% (generally, reliability scores greater than 80% are considered sufficient; Hartmann 1977; Watkins and Pacheco 2000; Borgeaud *et al.* 2016).

Social Network Measures

*General statements:* For each test, an edgelist was created with the trophallaxis time from individual *i* to individual *j* as the edge strength. The network was directed and weighted. We then calculated different individual and global measures using the ANTs (Sosa *et al.* 2018b) and igraph (Csardi and Nepusz 2006) R packages. We avoided measures that were not interpretable with the directionality of the edges (see Mersch, 2016; Sosa *et al.*, 2020; Sueur *et al.*, 2011); instead, we selected those that allowed us to make expectations based on our three aforementioned hypotheses. Table S1 shows the different indices and their associated expectations.

*Global network measures*: The global measures included maximum modularity, the centralisation index, global efficiency, and resilience. Maximum modularity is the strength of subgrouping or division of a network into modules or clusters (Newman 2004). It ranges from 0 to 1 with 0 corresponding to a network where all individuals are equally connected, and indices close to 1 corresponding to networks where the number and strengths of connections between individuals of different subgroups are low. The centralisation index captures the strength of centralisation of a network towards one or several individuals and how these central individuals gather relationships (Sueur *et al.* 2012; Pasquaretta *et al.* 2014). It ranges from 0 to 1 with 0 for corresponding to an equal network (*i.e.* all individuals are equally connected) and 1 for a star network (*i.e.* individuals are all connected to a single central individual). Global efficiency is the strength or speed of the exchange of entities—information or food—throughout the network (Latora and Marchiori 2001; Ek *et al.* 2015). Specifically, global efficiency equals N/(I×D) where N is the number of nodes, I is the number of edges and D is the network diameter. In other words, global efficiency indicates how quickly an entity is transmitted from the spreader (*i.e.* source) to the most peripheral individual in the group via the fewest number of connections. Global efficiency can range from 0 to 1, with more efficient networks having values closer to 1 (Romano *et al.*, 2018). Finally, we calculated resilience by dividing global efficiency by the centralisation index (Naug 2009; Puga-Gonzalez *et al.* 2019). Resilience assesses whether the strength or speed of the exchange of food can be maintained in the absence of central individuals, generally called bridges or hubs (Kitsak *et al.* 2010).

*Individual network measures:* The individual network measures included the degree (the number of edges of a node; *i.e.* the number of individuals giving or receiving trophallaxes from an ant), strength (the total time of trophallaxes of an ant; here we considered in-strength, the total time of trophallaxes received, and out-strength, the total time of trophallaxes given), betweenness (the number of shortest paths passing by a node; *i.e.* how many individuals an ant connects) and the clustering coefficient (whether individuals with which an ant exchanges food also exchange food). Detailed explanations of these different measures have been provided by previous reviews and books on animal networks (Whitehead 1997; Croft *et al.* 2008; Sueur *et al.* 2011; Sosa *et al.* 2020)

Statistical Analysis

*Food exchanges*: We first analysed the direction of trophallaxes between foragers and domestics. We used the ratio between the in-strength and out-strength to assess whether one caste gave more than it received (ratio>1) or received more than it gave (ratio<1). We used a student *t*-test to test for differences between the two castes. We used linear mixed models (package lme4 in R, Bates *et al.* 2014) to test for the effect of brood presence and colony size on global variables, such as the number of trophallaxes, the duration of trophallaxes and the forager/domestic ratio. Colony origin was included as a random effect. Data for all response variables were normally distributed (Shapiro-Wilk normality test, $W>0.93$, $p>0.074$). Therefore, all models were type-III ANOVAs using Satterthwaite's method and had normally distributed residuals.

*Global and individual network measures:* We then analysed the effect of brood presence, the number of individuals and the forager/domestic ratio for the global network measures (*e.g.* centralisation, global efficiency, modularity and resilience) as well as the caste (forager or domestic) for the individual network measures (*e.g.* degree, strength, betweenness and the clustering coefficient). Because data on the interactions and the network measures were not independent, we used Monte Carlo Markov Chain GLMM (package MCMCglmm in R, Hadfield 2010). This approach is a powerful and standard technique for comparing statistical models based on the original data observed to distributions of null models based on randomised data (Pasquaretta *et al.* 2014; Balasubramaniam *et al.* 2018; Sosa *et al.* 2018a). We ran MCMCglmm models for a minimum of 43,000 iterations after a burn-in of 3,000 to minimise autocorrelation and a thinning interval of 10 (*i.e.* one out of every 10 iterations in the Markov chain was used to estimate the posterior distribution of the parameters). We also assessed the robustness of the parameter estimates by checking the stability of the models. For all models, we assessed for approximate convergence of the MCMC chain (chain stability). We evaluated a final model's validity by assessing the distribution of residuals through residual normality distribution plots. The 95% credible intervals were calculated using Bayesian methods in the MCMCglmm package. We used a

Poisson law for the degree and strength and a zero-inflated Poisson law for the betweenness and clustering coefficient. Modularity followed a normal law while centralisation, global efficiency and resilience followed a log-normal law.

We then used individual network measures and the function PCA and HCPC of the FactormineR package in R to perform a hierarchical clustering analysis using principal components analysis to assess the presence of intermediary domestics. The HCPC function permitted us to determine whether some domestics acted as intermediaries (*i.e.* whether certain domestics had higher strength, in-strength, out-strength, degree and betweenness relative to other domestics). We also performed Pearson correlations between the different individual network measures. All tests were conducted on R 3.6 (R Development Core Team 2009). The significance level was set at 0.05. Results are shown as mean ± SD [median].

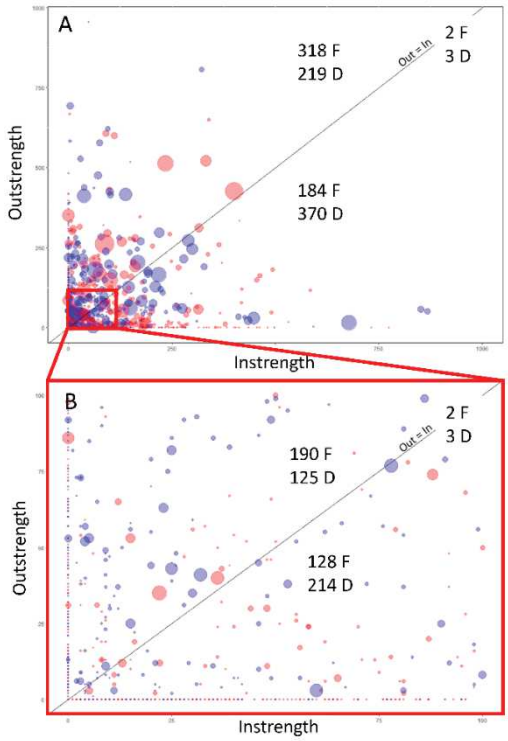


**Figure3: Out-strength (total duration of giving food) by in-strength (total duration of receiving food), (A)** for all the dataset, **(B)** for trophallaxes fewer than 100 frames. Blue dots are for foragers whilst red dots are for domestics. Size of the dots represents betweenness. The numbers indicate the foragers (F) or the domestics, on the line (Ratio=1), under the line (Ratio<1) or above the line (Ratio>1).

*Results*

*Food exchange*: At the individual level, foragers spent more time giving than receiving food (t=75.7, df=33, p <0.0001, table 1). The opposite pattern was observed for domestics (t=-138.1, df=33, p <0.0001) (fig. 3). However, figure 3 shows a high level of variability among both foragers and domestics: some foragers spent more time receiving than giving and some domestics spent more time giving than receiving. This result was also recovered at the global (fig. 3) and test levels (fig. S2).

At the colony level, the mean duration of trophallaxes (31.7 ± 12.8 sec, corrected by colony size) was not influenced by the presence of broods (df=29.9, t value=1.34, p=0.189) nor by colony size (df=29.9, t value=2.03, p=0.051, fig. S3). The total number of trophallaxes (77.6 ± 49.1) significantly increased with colony size (df=30, t value=7.62, p <0.0001, fig. S4a) but did not change significantly with brood presence (df=30, t value=1.32, p=0.197). However, the number of trophallaxes corrected by colony size (mean number: 2.04 ±1.13) did not change with colony size (df=30, t value=0.51, p=0.613) showing that the effect

was more or less linear. This corrected number of trophallaxes did not change with brood presence (df=30, t value=1.31, p=0.197). Lastly, the forager/domestic ratio (0.47 ± 0.21) was not influenced by colony size (df=27.7, t value=1.02, p=0.315) but was increased when broods were present (df=28.6, t value=3.07, p=0.005, fig. S4b).

Table 1: Mean ± standard deviation of individual network measures according to brood (presence/absence) and then caste (foragers/domestics). The relative difference between foragers and domestics is also indicated.

| **Brood** | **Presence** | | | **Absence** | | |
|---|---|---|---|---|---|---|
| **Caste** | **Forager** | **Domestic** | **Diff(F-D)** | **Forager** | **Domestic** | **Diff(F-D)** |
| **Degree** | 3.2 ± 2.2 | 2.4 ± 1.8 | 0.8 | 4.1 ± 2.6 | 3.2 ± 2.2 | 0.9 |
| **Instrength** | 38.7 ± 68.7 | 84.2 ± 111.5 | -45.5 | 90.0 ± 162.7 | 94.8 ± 123.7 | -4.8 |
| **Outstrength** | 77.5 ± 106.2 | 43.9 ± 83.3 | 33.6 | 113.8 ± 155 ± 2 | 82.3 ± 128.5 | 31.5 |
| **Strength** | 123.8 ± 146.8 | 120.5 ± 128.9 | 3.3 | 203.8 ± 223.9 | 177.1 ± 188.4 | 26.7 |
| **Betweenness** | 30.1 ± 81.1 | 18.5 ± 62.0 | 11.6 | 55.8 ± 120.5 | 35.2 ± 126.1 | 20.6 |
| **Clust. coeff.** | 0.08 ± 0.17 | 0.06 ± 0.17 | 0.02 | 0.19 ± 0.26 | 0.16 ± 0.26 | 0.03 |

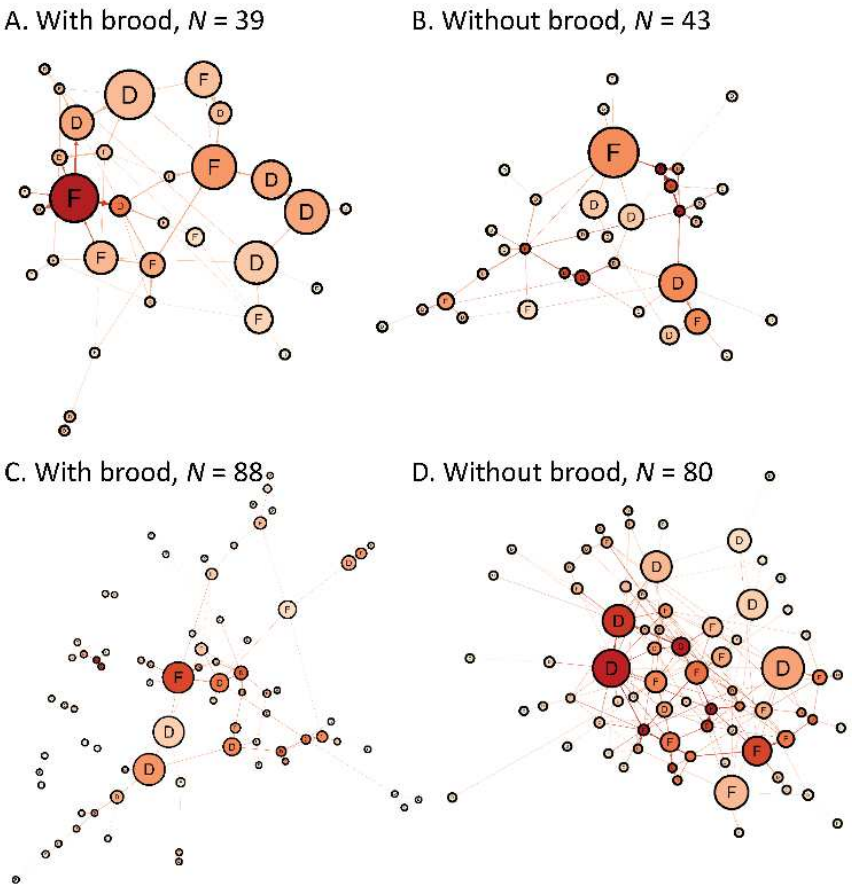


**Figure 4: Illustrations of four trophallaxis networks,** with and without brood and with two comparable sizes. D indicates domestics whilst F is for foragers. The size of the nodes corresponds to betweenness (the bigger, the higher) whilst colour fits with the degree (the redder, the higher). Graphs were drawn with Gephi 0.9.2 (Bastian *et al.*, 2009) using the force Atlas 2 package for the spatial visualization.

*Global network measures:* Four illustrations of trophallaxis networks are shown in figure 4. The number of ants negatively influenced network centralisation (l-95% CI=-0.017, u-95% CI=-0.008, p <0.001, fig. 5a) and global efficiency (l-95% CI=-0.02, u-95% CI=-0.0001, p=0.04, fig. 5c), positively influenced modularity (l-95% CI=-0.001, u-95% CI=0.005, p=0.002, fig. 5b) and did not impact resilience (l-95% CI=-0.009, u-95% CI=0.013, p=0.626). However, resilience increased in the presence of broods (l-95% CI=-1.51, u-95% CI=-0.14, p=0.026, fig. 5d); in contrast, brood presence had no effect on the other global network measures (centralisation: l-95% CI=-2.40, u-95% CI=-1.47, p=0.116; modularity: l-95% CI=-0.125, u-95% CI=0.11, p=0.882; global efficiency: l-95% CI=-1.20, u-95% CI=0.013, p=0.059). The forager/domestic ratio did not influence the global measures (p>0.225; fig. S5). Results of all statistical tests are shown in the supplementary material.

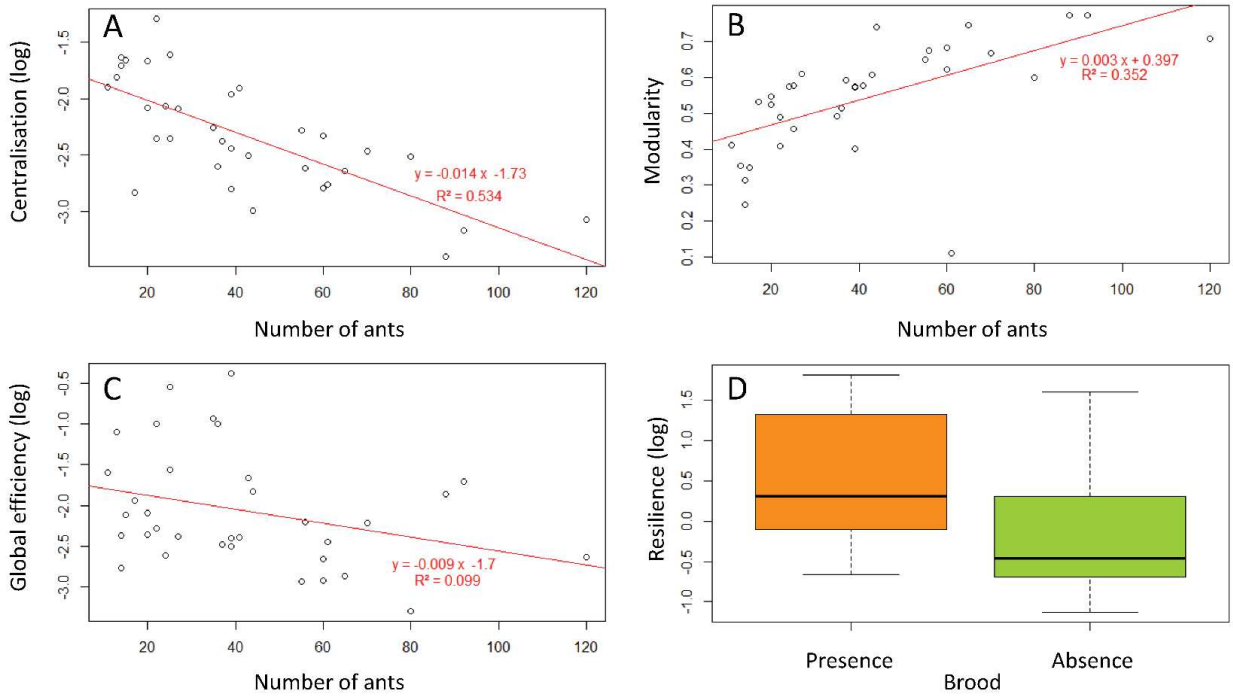


**Figure 5: Effects of factors on global network measures.** **(A)** Number of ants on centralisation. **(B)** Number of ants on modularity. **(C)** Number of ants on global efficiency. **(D)** Presence of Brood on network resilience.

*Individual network measures:* Distributions of individual network measures (table 1) are shown in fig. S6 (supplementary material). Local peaks were observed for foragers and domestics in the distributions for degree, out-strength and clustering coefficient. The degree decreased in the presence of broods (l-95% CI=-0.057, u-95% CI=0.293, p=0.001) and decreased with colony size (l-95% CI=-0.004, u-95% CI=-0.0004, p=0.015). Domestics had a lower degree than foragers (l-95% CI=-0.370, u-95% CI=-0.197, p <0.0001). Strength was negatively influenced by the forager/domestic ratio (l-95% CI=-3.061, u-95% CI= –1.576, p <0.0001). Surprisingly, caste did not influence strength. Foragers had a higher betweenness than domestics (l-95% CI=-0.590, u-95% CI=-0.067, p=0.013). Betweenness also increased with the

forager/domestic ratio (l-95% CI=-0.590, u-95% CI=-0.066, p=0.013) and with colony size (l-95% CI=0.016, u-95% CI=0.028, p <0.0001) but decreased in the presence of broods (l-95% CI=-0.284, u-95% CI=-0.066, p=0.012). Similar to betweenness, the clustering coefficient was lower in the presence of broods (l-95% CI=-0.014, u-95% CI=0.083, p=0.002) and decreased with colony size (l-95% CI=-0.002, u-95% CI=-0.001, p <0.0001). All other variables had no effect on the clustering coefficient. Results of all statistical tests are provided in the supplementary material.

A hierarchical clustering analysis following a principal components analysis confirmed the presence of three clusters in the domestics. Details of this analysis are provided in the supplementary material (fig. S7). All individual network measures, as well as brood presence, colony size and the ratio foragers/domestics, significantly affected the clustering. Cluster 3 was composed of domestics with higher strength, in-strength, out-strength, degree and betweenness compared with the other two clusters identified.

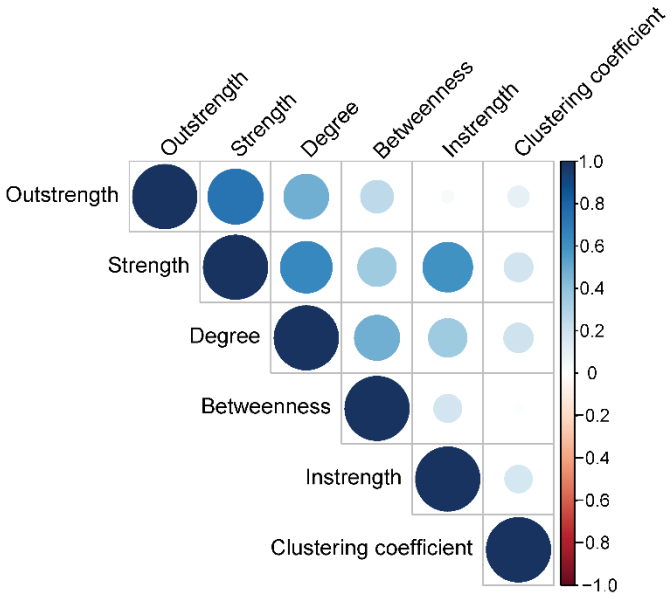


**Figure 6: Correlations matrices between the different individual network measures**

Correlations between individual network properties showed that only in-strength and out-strength (r=0.03; p=0.246, n=1096), as well as betweenness and the clustering coefficient (r=0.02; p=0.513, n=1096), were not correlated. All other indices were correlated (0.10 <r <0.73; p <0.0005, n=1096, figure 6, table S2)

*Discussion*

The goal of this study was to understand the organisation of trophallaxes in black garden ants, assess the topology of the trophallaxis network and characterise variation in the centralities among foragers and domestics. Consistent with expectation, our results showed that the trophallaxis network in black garden ants was not random and that the directionality of this network was oriented from foragers to domestics. The high betweenness and out-strength values indicated that intermediary domestics existed. In addition, local peaks and clusters were observed in the distribution of some domestic centralities. These intermediary individuals provided the link between the source (foragers) and the final destination of food. This division of labour resulted in a food dissemination chain in the form of a hierarchical network. We also observed high variability among individuals, consistent with previous studies that have examined the distribution of activities (specifically, the percentage of time

foraging, distributing food or working inside colonies on a per-individual basis) among workers (Kolmes and Sommeijer 1992; Dornhaus *et al.* 2009; Tenczar *et al.* 2014; Quevillon *et al.* 2015; Bles *et al.* 2018).

Variation in Global Network Values: The Rise of Intermediate Workers

The mean duration of a trophallaxis (tendency) and the total number of trophallaxes increase with colony size and were not influenced by the presence of a brood. Moreover, the forager/domestic ratio was not influenced by colony size (as was found in Dornhaus *et al.*, 2009 but in the presence of a queen) but increased in the presence of a brood. This finding demonstrates the behavioural plasticity of ants and their capacity to respond to changes in the demand for food by the colony, regardless of its size and composition (Portha *et al.* 2002; Mailleux *et al.* 2011; Tenczar *et al.* 2014). When broods are present during experiments, domestics can become foragers to address the increased protein needs of the brood (Lee Cassill and Tschinkel 1999; Dussutour and Simpson 2008, 2009). In contrast, when broods are absent, foragers can become domestics (Sendova-Franks and Franks 1993; Huang and Robinson 1996; Amdam 2005; Baker *et al.* 2012). This adaptability is also supported by our global network measures. Network efficiency is an important network parameter contributing to the success of colonies (Sendova-Franks *et al.* 2010; Waters and Fewell 2012; Stroeymeyt *et al.* 2018). Colony members are known to modulate their interactions, and thereby the network, based on food availability and the needs of the colony (Sendova-Franks *et al.* 2010; Pinter-Wollman *et al.* 2011), the spread of disease (Stroeymeyt *et al.* 2018) and group/colony size (Pasquaretta *et al.*, 2014; Romano *et al.*, 2018). The relationship between network efficiency and network size is non-linear, as there are different peaks of efficiency at different values of modularity based on group size (Romano *et al.*, 2018). This non-linearity is interpreted as an adaptive mechanism for optimising the social transmission of information and impeding the transmission of pathogens (Waters and Fewell 2012; Nunn *et al.* 2015; Sueur *et al.* 2019; Romano *et al.* 2020).

We found that global efficiency decreases with colony size. However, resilience—the ratio between efficiency and centralisation—was stable with colony size but increased in the presence of a brood, presumably in response to the needs of the larvae. Improving or maintaining network resilience while ensuring reproduction, can increase the ability of the colony to recover from cascading failures (*i.e.* breaks in the transmission chain, Wang and Xiao 2016). Moreover, modularity increases with colony size. Interestingly, caste-specific adaptations to colony size might also occur, and the role of intermediary domestics may be crucial. For example, intermediary domestics may stabilise the link between foragers and domestics, promoting network resilience with increasing group size. Indeed, the hierarchical clustering analysis revealed the presence of a cluster of intermediary domestics with higher strength, in-strength, out-strength, degree and betweenness compared with other domestics. In sum,

our global measures indicate—similar to the findings of Stroeymeyt *et al.* (2018) for disease—that the food dissemination process and interactions at the colony level are highly flexible and depend on colony size and needs. All subcolonies used had been in the lab for 18 months to decrease the potential effect of temporal polyethism. The possibility of a potential age effect was also minimised by the fact that we conducted multiple tests with different colony sizes as well as the 10-day buffer that we provided to colonies before scoring interactions. Moreover, ants can continually adapt to the needs of colonies as individuals change castes—even to changes from foragers to domestics (Sendova-Franks and Franks 1993; Huang and Robinson 1996; Baker *et al.* 2012). Thus, we believe that the effect of temporal polyethism on our findings was negligible.

Variation in Individual Network Values: Towards a Decentralised and Hierarchical Organisation

We found that foragers had higher degree and betweenness but the same strength and clustering coefficient as domestics (see supplementary material for detailed statistics). This pattern is consistent with foragers giving more than they received and domestics receiving more than they gave (Quevillon *et al.* 2015). Because foragers gave more food to more ants than domestics did, they also had a higher betweenness (degree and betweenness are correlated). Thus, the degree, betweenness and out-strength values clearly indicate that foragers were the ones distributing food in the network, supporting the centralised network hypothesis. This finding was further confirmed by the fact that betweenness increased with the forager/domestic ratio. Specifically, foragers became more important for food exchange as the number of intermediaries decreased, and when intermediaries were few, the number of foragers increased. However, the fact that foragers had a similar strength and clustering coefficient compared with domestics suggested that there were connections between domestics and that the network was not highly centralised, allowing the trophallaxis network to retain its adaptability and resilience. Decentralisation was supported by the decrease in strength (but not degree) as the forager/domestic ratio increased. Specifically, foragers gave less per trophallaxis when their number was higher, or domestics served as intermediaries by giving more when the number of foragers was low. While the trophallaxis network was not randomly organised, the presence of intermediary ants, confirmed by the local peaks and the hierarchical clustering analysis, indicated that the form of the trophallaxis network was most consistent with the hierarchical network hypothesis. Trophallaxes were clearly directed in different steps and levels, which made the networks hierarchical.

Social Network and Brood

The presence of a brood did not influence individual strength. Strength was quite stable given that no parameter, except the forager/domestic ratio, affected it. Strength reflects the duration of trophallaxis of an individual, and this should depend more strongly on intrinsic characteristics (Greenwald *et al.*,

2018), such as the quantity of food present in the social stomach. The crop capacity is generally stable between individuals measured at distinct foraging events (Greenwald *et al.*, 2018). Thus, foragers should always transfer stable quantities of food but some might occasionally give large quantities of food to domestics and at other times several small quantities of food. Our results highlight that the pattern of food distribution is independent of colony size and the presence of a brood. In contrast to expectation, the degree and betweenness of foragers and intermediary domestics did not increase in the presence of a brood. This finding may stem from increases in the number of foragers. As a consequence of such a decentralised organisation, the degree and betweenness *per* individual decreases. Thus, a more decentralised and resilient network with intermediary domestics may arise, preventing food exchange from being concentrated to a small number of foragers. The change in the forager/domestic ratio in the presence of a brood indicated that the system balances foraging and caregiving effort (Schafer *et al.* 2006; Mailleux *et al.* 2011). The mechanisms underlying the responses of the system, namely behavioural plasticity of the workers, reinforce the hypothesis that the network was more hierarchical than centralised (Middleton and Latty 2016).

Social Network and Colony Size

An increase in colony size negatively influenced the degree and the clustering coefficient of ants, positively affected their betweenness and had no effect on their strength. These results corroborate the findings of several previous studies (Naug 2009; Pasquaretta *et al.* 2014; Puga-Gonzalez and Sueur 2017). As colony size increases, the probability of interactions between each pair of ants inherently decreases, resulting in a decrease in network density and as consequence, in the clustering coefficient. However, in larger colonies, as the network becomes more centralised, the mean individual betweenness should decrease. This expectation should hold if interactions are more or less random but not directed towards specific individuals as trophallaxes often are. Thus, one explanation that might explain our finding of betweenness decreasing with colony size is that in larger colonies, some individuals—either foragers or intermediary domestics—become more important in the transfer of food, allowing us to reject the random network hypothesis. Because some domestics had an out-strength/in-strength ratio greater than one (figure 3), our data most strongly support the hierarchical network hypothesis.

Conclusion

Behavioural flexibility and decentralised control (the presence of several individuals per caste) are parameters characterising the organisational resilience of ants (Middleton and Latty 2016). Several studies have already demonstrated the presence of decentralised but hierarchical networks in mammals (Hill *et al.* 2008). Various hierarchical networks have been described in nature ranging from protein complexes (Ravasz *et al.* 2002), to neural networks (Chatterjee and Sinha 2007; Clune *et al.* 2013), to

animal groups (Hill *et al.* 2008) and to organisation in social insects (Linksvayer *et al.* 2012). Hierarchical networks are more resilient than centralised networks but less costly in terms of connections (time to find partners and exchanges) than complete networks (Banavar *et al.* 1999; Guimera *et al.* 2001; Ravasz and Barabási 2003; Barabasi *et al.* 2003; Bode *et al.* 2010). Changes in our global network measures, such as efficiency, centralisation or resilience, may ultimately result in increases in group performance as mentioned by Sueur (2012) and described by Fontanari and Rodrigues (2016). The two latter authors hypothesised that the collective cognition behind the complex systems built by social insects suggests that the topology of social networks is selected to optimise problem-solving competence at the group level. However, although individuals are selected and not the group or networks, feedbacks exist between these two levels (Farine *et al.*, 2015; Fisher & McAdam, 2017). This process of multilevel selection previously described as "collective social niche construction" by Sueur *et al.* (2019) deserves increased attention. Indeed, study of this process promises to enhance our understanding of the evolutionary mechanisms contributing to the emergence of complex systems.

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**Data availability:**

Supplementary data are available online at ncloud2.zaclys.com/index.php/s/nLAdQ8Qrm43aAfi (Appendix 2).

# Appendix 3 | Chapter in the Encyclopedia of social insects: Lasius

## Lasius

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**Examples of four *Lasius* species** to show the relatively generalised body form. (**a**) *L. neoniger* queen, Illinois, USA. (**b**) *L. americanus* worker, Illinois, USA. (**c**) *L. latipes* queen, Illinois, USA. (**d**) Two-winged females (bigger), one-winged male, and workers of *L. nearcticus*, New York, USA. (All photos © Alex Wild)

*Synonyms*

Donisthorpea (Morice and Durrant, 1915) and Tylolasius (Zhang, 1989) are junior synonyms, not in use anymore. The former genus *Acanthomyops* is now a subgenus included in *Lasius*.

*Introduction*

*Lasius* (Fabricius, 1804) is an ant genus (Family: Formicidae, Subfamily: Formicinae,Tribe: Lasiini) including 115 extant species, 1 extant subspecies, 22 fossil species and 1 fossil subspecies. (Bolton 2018)

The genus name stems from the Greek λάσιος (lasios), meaning "hairy". Morphologically, *Lasius* species are characterised by the following attributes. The antennae are made of 12 segments and are filiform or broaden gradually towards apex but do not form a well-differentiated club. The maxillary and labial palps have respectively six and four segments. Depending on the species, there are six to ten teeth on the masticatory margin and zero to one on the basal one. The mesotibia and metatibia have both one simple spur. Eyes are visible. Scrobes and sting are absent from this genus. The difference in size

between workers and queens excepted, the caste-polymorphism is not very marked in *Lasius* species. (Bolton 2003)

If you live in North America or Europe, you are very likely to have already encountered ants from the genus *Lasius*, and on more than one occasion. Perhaps driven by this conspicuousness, species of the genus *Lasius* have been extensively studied since the 18th century, leading to a good knowledge of their taxonomy and biology. These studies relate to very different fields: from the evolution of social parasitism, to mutualistic relationships (with aphids and lepidopteran), to the invasion of the species *L. neglectus* in Europe, to soil ecology (*e.g. Lasius flavus*). Finally, *L. niger*, the type species of the genus, is used in various experiments aiming at understanding the mechanisms of ageing.

*Geographic distribution and habitat*

From south-eastern Alaska to southern Himalaya, and from northern Scandinavia to northern Africa, *Lasius* is a widespread genus in the northern hemisphere. Although the repartition area is limited to the Holarctic biogeographic realm, *Lasius* species face diverse habitat and climates: *e.g.* high-altitude grasslands, woodlands, meadows, fields, sidewalks. *L. niger* is the most commonly found ant species in urban areas. In more tropical climate, they limit their distribution to temperate vegetation at higher elevation.

Species from the *Lasius* genus are known to cope greatly with open habitats. For instance, *L. neoniger* is pervasive in golf greens where it builds little unpopular mounds. From a less anthropocentric point of view, it must be acknowledged that ants benefit the soil ecology by modifying the composition, the texture and fostering infiltration. Within the *Lasius* genus, *L. niger* and *L. flavus* are described as pioneer species, able to face unstable and disturbed habitat. By digging the anthill and carting food inside and outside, they modify carbon, nitrogen, and phosphorus cycles, as well as cadmium concentration. *Lasius* species demonstrate that ant ecological roles are multiple.

*Nesting habits*

Where to establish the colony?

Depending on the species, nests can be wood-based (*e.g. L. bruneus*, *L. fuliginosus)* or directly in the ground (*e.g. L. niger, L. neoniger*, *L. flavus*). While jet black ants (*L. fuliginosus*) and *L. spathepus* build an elaborate nest in old hollow trees with a mixture of saliva, chewed wood and honeydew, *L. neglectus* ants merely nest in the topsoil under leaf litter or stones, and sometimes even inside electrical devices.

Who will establish the colony?

The number of queens (also called gynes) varies between species. Almost all *Lasius* mature colonies have only one queen (strictly monogynous). Depending on the species, queens can be fertilised by one (monoandrous colony) or many (polyandrous colony) males. New queens store enough amounts of nutrients in their native colony to found an independent colony, rearing the first batch of workers alone the first few months. Nevertheless, in some of these monogynous species (*e.g.*, *L. emarginatus*, *L. niger*, *L. pallitarsis*), foundress queens cooperate to build a common nest. After the first workers hatched, only one queen will remain, because either the workers kill the less fertile ones, or because the dominant queen expels or kills the other females. The colony, primarily polygynous, becomes then monogynous. This phenomenon, called pleometrosis, does not mandatorily occur in a species: only 25% of European *L. niger* colonies have a pleometrosis-based foundation, while the remaining 75% are strictly monogynous. Oligogynous and polygynous colonies have been observed only in few species: *L. spathepus*, *L. brunneus*, *L. flavus*, *L. turcicus, L. fuliginosus* and *L. sakagamii*. The occurrence of polyandry, polygyny and pleiometrosis in a species depend on the local conditions.

How many nests?

Polydomy is a social structure where distinct nests regularly interchange workers and brood. Polydomy is facultative in *Lasius* species and has been reported in *L. flavus*, *L. neoniger*, *L. minutus*, *L. neglectus and L. sakagamii*. *Polydomous* colonies of *L. alienus* and *L. neoniger* occupy different nests in winter and during the active season, leading to a seasonal polydomy. Polygyny is associated with polydomy in *L. minutus*, *L. negecltus*, *L. sakagamii*, and with monogyny in the three other polydomous *Lasius* species. The social structure plasticity is perfectly illustrated in *L. flavus*, where, even within a 100m² area, colonies can differ by their number of queens and nests (Steinmeyer *et al.* 2012).

*Social parasitism*

Most of ant colonies are founded by one or several queens that take care of the eggs before the first workers emerge and take over the nursing task. However, in less than 2% of ant species (Buschinger 2009), queens and/or workers are unable to provide care to the eggs and larvae. The colony must therefore find a host colony – from the same or other species. This relation is generally referred to as social parasitism. *Lasius* parasitic species display only a temporary social parasitism. The parasite young queen is adopted and the host queen killed by the intruder or by her own workers. The host workers rear the brood of the new queen and are thus progressively replaced by workers of the parasite species. *L. umbratus* takes the social parasitism relation one step further. This species parasitizes other species of the same genus, *L. niger* or *L. alienus*. However, being a parasite does not prevent it from being parasitised by a third species of the *Lasius* genus, *L. fuliginosus*. By parasitizing a parasite, *L. fuliginosus*

earns the name of hyper-parasite. However, *L. fuliginosus* is not an obligate parasite of *L. umbratus* and can for instance be found in *L. niger*'s nests. A more complete list of parasitic species in *Lasius* and other genera, as well as an open discussion about the evolutionary origins of social parasitism in ants, are available in the review of Janda et al. (Janda *et al.* 2004) and in references therein.

*Caste pattern*

As stated above, there is a strong caste dimorphism between the queens and workers. However, workers are indistinguishable from each other. The division of labour is thus more age-based than morphology-based and high behavioural plasticity is observed (Robinson 1992).

*Mutualistic interactions*

With Aphids

Aphid farming is widespread among *Lasius* species, although this activity also occurs in other genera *(see ant-Hemiptera associations)*. Ants feed on the honeydew excreted by Aphids, which provides a carbohydrate-rich solution. However, the Aphids also benefit from the interaction (mutualism). By tending *Aphis fabae*, *L. niger* ants prevent the Aphids from predation, increase their excretion and reproductive rates, modify the dispersal pattern along the plant. Workers of the ants *L. productus* and *L. neoniger* do not differentiate the Aphid's egg from their own in the way they tend them, increasing substantially their survival. *L. niger* is able to discriminate Aphid species according to their cuticular hydrocarbons. This recognition mechanism prevents useless efforts in rearing non-trophobiont Aphids.

With Lepidoptera

*Plebejus argus* lay their eggs in the neighbouring of colonies of *L. niger* and *L. alienus*. Driven by pheromonal signaling, the ants bring the eggs inside the colony where they will be protected from extrinsic mortality. The ants benefit from this interaction by feeding on a solution comprised of saccharine and amino acids, produced by a gland on the larva's back. At the imago's emergence, the butterfly leaves the nest. (Seymour *et al.* 2003).

*Ecological status*

Endangered species

Only three species belonging to the *Lasius* genus are indexed in the IUCN red list among which two are listed under the former genus *Acanthomyops* instead of *Lasius*. *L. reginae*, *L.* (*Acanthomyops*) latipes. *L.* (*Acanthomyops*) *murphyi* are all three classified as vulnerable.

*L. balearicus* the sole endemic *Lasius* species from Mediterranean islands (Talavera *et al.* 2015) has been recently described as a new species and, according to the authors, should be already classified as endangered (EN) in the IUCN Red List because of climate change.

Lasius neglectus: an invasive species

Like other invasive species, *L. neglectus* is organised in a polygynous supercolony structure. Within the interconnected nests of the supercolony, the population can reach and even exceed 35 500 individuals. Their presence leads to exclude other ant species and decreases the species richness of other insects, notably Isopoda. Furthermore, the countless Aphids that the supercolony needs to maintain to feed on results in damaging the trees. This invasive species would come from Asia Minor and it is now widespread in Northern and Western Europe. Since it does not require warm temperatures to thrive, it can spread out into temperate areas with ease. All this appoints *L. neglectus* as a potential issue in conservation ecology, like the Argentine ant, *Linepithema humile* (Cremer *et al.* 2008).

*A type species at the edge of ageing research*

Social insects emerge as a remarkable model to study ageing (Parker 2010), notably by their overall longevity and the difference in lifespan between the castes. Two *Lasius* species stand out by their maximal lifespan recorded in the protected conditions of a laboratory (Keller 1998): L. flavus (22,5 years) and L. niger (28,5 years). The latter has been the subject of many recent studies trying to disentangle the effect of age and caste on the phenotype, either by using a global genomics approach or by targeting specific mechanisms such as telomere length and telomerase activity, or oxidative damages. *L. niger* is so far the only ant species where such questions have been asked so thoroughly. This final point and the previous ones emphasize the diversity of research conducted on *Lasius* species, from ecology to the molecular bases of ageing.

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**Abstract** | Coevolution of sociality and ageing in animal societies.

In order to improve our knowledge of the mechanisms of ageing in animals, the main objective of the thesis was to understand the modulation of such mechanisms by the individual social role, within different social organisations. This objective thus addresses two main questions: i) describing the covariation of the degree of social complexity with ageing patterns; ii) highlighting the underlying cellular and molecular processes. Thanks to complementary and diversified studies (behavioural observations, dosage of the oxidative balance, qPCR measurement of telomere length, proteomics, metabolomics), the present thesis showed that sociality plays a role on ageing at many levels. In the zebra finch, social stress caused by aggression of the conspecifics induces oxidative stress and reduces telomere length in adults. In the sociable weaver, the social environment is of crucial importance during pre- and post-hatch development on the medium-term survival of the chicks. Finally, in ants, we were able to show a positive relationship between the degree of sociality and maximum potential life span: this link was caste specific, being only significant for the most social queens. This is in line with a recent review by Lucas and Keller (2020) which concluded that the benefits of sociality are most sensitive for high levels of sociality and particularly in reproductive individuals. With regard to the molecular mechanisms of ageing, we were able to establish a causal chain between social stress, oxidative response and telomere erosion in zebra finches. The role of telomeres as a predictor of offspring survival has been confirmed (over at least 5 years) in the sociable weaver, a cooperative breeder bird. However, this link was not true in queen ants where the longest lived were those with the shortest telomeres. The co-evolution of anti-cancer mechanisms and longevity seems to be conserved since similar strategies are found in taxa as diverse as ants and rodents. On the other hand, and contrary to previous studies conducted on ants, we found that oxidative stress might be a marker of individual ageing. We suggest that the proxies of oxidative stress used so far in ants have been misleading or at least incomplete. Thus, understanding the physiological ageing particularities of ants and other social insects might require finding new relevant and specific markers. Finally, the sirtuins and mTOR signalling pathways, key precursors of which we have detected in ants, are molecular crossroads capable of activating or inhibiting cellular metabolism depending on the cell energy state. According to the studies carried out to date, these signalling pathways are among the first to be able to slow down the effects of ageing and extend life expectancy. However, specific studies need to be carried out to understand their fine regulation and thus assess the universality of these mechanisms in animal ageing. Based on our findings, we propose three points to be further addressed to better understand the mechanisms of ageing in social insects: i) the setup of experiments testing the effectiveness of energy trade-offs involving immunity or digestion metabolism; ii) measuring the telomerase activity among castes of various species in order to explore the telomere and telomere independent roles played by this enzyme in ageing; iii) the need to think about individual longitudinal follow-up and to study wild populations, after the first necessary stages in laboratory.

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