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Author for correspondence:
Tobias Pamminger
e-mail: t.pamminger@sussex.ac.uk

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Pleiotropic effects of juvenile hormone in ant queens and the escape from the reproduction–immunocompetence trade-off

Tobias Pamminger, David Treanor and William O. H. Hughes

School of Life Sciences, University of Sussex, Brighton BN1 9QG, UK

The ubiquitous trade-off between survival and costly reproduction is one of the most fundamental constraints governing life-history evolution. In numerous animals, gonadotropic hormones antagonistically suppressing immunocompetence cause this trade-off. The queens of many social insects defy the reproduction–survival trade-off, achieving both an extraordinarily long life and high reproductive output, but how they achieve this is unknown. Here we show experimentally, by integrating quantification of gene expression, physiology and behaviour, that the long-lived queens of the ant *Lasius niger* have escaped the reproduction–immunocompetence trade-off by decoupling the effects of a key endocrine regulator of fertility and immunocompetence in solitary insects, juvenile hormone (JH). This modification of the regulatory architecture enables queens to sustain a high reproductive output without elevated JH titres and suppressed immunocompetence, providing an escape from the reproduction–immunocompetence trade-off that may contribute to the extraordinary lifespan of many social insect queens.

1. Introduction

Reproduction carries a survival cost in a wide range of taxa [1]. This fundamental phenomenon, termed the reproduction–survival (RS) trade-off, has been observed both within and between species [1,2], and is thought to play a key role in shaping the evolution of life-history strategies [3]. The universal occurrence of this trade-off, ranging from insects to mammals, implies a common underlying mechanism, but despite substantial efforts the physiological underpinning of this antagonistic trait association remains poorly understood [2,4,5]. Traditionally, the RS trade-off has been interpreted in the context of competitive resource allocation, in which energy invested in reproduction is withdrawn from somatic maintenance and vice versa causing the observed life-history continuum [6]. In recent years, this interpretation has been challenged on experimental grounds by demonstrations that reproduction and survival can be experimentally decoupled [6]. Owing to their pleiotropic nature and central regulatory function, hormones have emerged as prime candidates mediating trade-offs on a proximate level [5,7]. In insects, juvenile hormone (JH) occupies such a central role, regulating both reproductive and survival-relevant processes [8,9]. JH in most adult insects acts as a gonadotropin, stimulating the expression of *vitellogenin* (*Vg*), the precursor of the yolk protein found in the eggs [10,11]. In addition, JH acts as a potent immune suppressor in some insects, regulating the central pathways of insect innate immunity [12–15], suggesting that the resulting JH-mediated immunocompetence–reproduction trade-off might underlie the RS trade-off in insects [5,7,12,16].

In contrast to solitary insects, the reproductives (queens) in some eusocial insects exhibit both extraordinarily long lifespans, which can be 100 times longer than the average solitary insect [17–20], and phenomenal reproductive capacities, with the ability to lay over 120 000 eggs per month in some species [21,22]. Although longevity and reproduction depend on a wide variety of

factors, the extraordinary longevity and reproductive output of eusocial insect queens has led to it being suggested that they may have somehow escaped the JH-mediated RS trade-off [23]. We hypothesize that queens of some social insects have escaped this trade-off by releasing JH of its stimulatory gonadotropic function, enabling the maintenance of its central regulatory (suppressive) role in the innate immune system. Using experimental endocrine manipulation by application of the JH analogue (JHa) methoprene, we investigate if JH still acts as a gonadotropin in queens of the long-lived (up to 28 years; [20]) ant species *Lasius niger* [24]. We integrate data on egg production (experiment 1), with maternal behaviour, brood development and gene expression analysis (experiment 2) to capture the expected pleiotropic effects of JH in the reproductive process. We also determine the effects of JH on the innate immune system of *L. niger* queens (experiment 3) by examining its regulatory role in three key pathways (phenoloxidase, PO) and pro-phenoloxidase (PPO) activity, expression of the anti-microbial peptide *defensin* (*def*), and of the protease *cathepsinl* (*cath*)), and by experimentally challenging JHa-treated *L. niger* queens with the fungal pathogen *Metarhizium* to test whether an endocrine suppression of the innate immune system carries fitness costs.

2. Material and methods

Full methodological details are provided in electronic supplementary material (S1). In brief, newly mated queens of *L. niger* were collected during their mating flight on 17 July 2014 on the campus of the University of Sussex, Brighton, UK (50°52′02.8″ N 0°05′09.6″ W). Queens are produced synchronously in this species, so all queens were approximately the same age, and queens once mated found their colony claustrally and do not remate. In all experiments, queens were randomly assigned to a treatment group. Neither their head width (in all experiments), brood items (larvae and pupae in experiments 2 and 3) nor number of workers (experiment 3) differed between the treatment groups at the start of each experiment (see Results). In all experiments, the queens were inspected daily to detect potential differences in survival rates between treatments. We used methoprene (PESTANAL®, Sigma-Aldrich), a synthetic JHa, to experimentally simulate an increased JH titre in *L. niger* queens. In all cases, a 1 µl (1.1 µg per 1 µl) dose was topically applied to the abdomen five times a week over the course of the experiments. This dose was chosen based on previous JH studies in *L. niger* queens [24] and confirmed as being biological relevant during a dose–response experiment. We found this concentration to maximize the expected physiological effect while minimizing the JHa exposure (see electronic supplementary material for details). This dose was not found to increase mortality during the duration of the experiments and is well within the range used in previous studies of insects, including social insects [25–29].

3. Experiment 1: egg laying rate

Sixty *L. niger* queens were randomly assigned to one of three treatments groups. Queens in the first group were treated with JHa, those in the second group received 1 µl of acetone as a solvent control (CoA), and those in the third group were disturbed with a pipette once a day as a handling control (CoH; treatment did not affect survival—see Results). Three days after the start of the experiment, all queens were transferred (without their first egg clutch) to new glass test tubes and the numbers of eggs laid over the following two weeks was counted.

4. Experiment 2: queen maternal behaviour, reproductive physiology and immunocompetence

Because neither a preliminary experiment (T Pamminger 2014, unpublished data) nor experiment 1 indicated any difference between the acetone- and handling control, the latter was dropped from both subsequent experiments. In experiment 2, 156 queens were randomly assigned to either the JHa or CoA group and treated over a period of three weeks (treatment did not affect survival—see Results). To determine the effect of treatment on activity, we randomly selected 35 queens per treatment after 14 days of treatment and recorded the amount of time the queens spent moving (activity) or performing maternal behaviour (cleaning, guarding or feeding brood) in two 2 min observation periods. To determine the effect of treatment on brood development, we collected larvae at the end of the experiment from all colonies in which enough larvae were present and analysed the protein content on a Nanodrop 2000® [30]. To determine reproductive capacity, we recorded the status (proportion vitellogenic and non-vitellogenic eggs) of the most distal oocyte of 10 randomly selected ovarioles in 20 queens per treatment. To determine immunocompetence, we randomly selected 40 queens per treatment group, collected haemolymph from their thoraxes using a sterile glass capillary (1–4 µl haemolymph per queen) diluted the samples 1 : 40 in cacodylate buffer and used 15 µl of the diluted samples to quantify levels of the PO and PPO [31].

In addition, we randomly selected 15 queens per treatment to determine the expression of the central reproductive gene *vitellogenin*, and the two immune genes *defensin* and *cathepsinl*. The frozen gaster of each queen was homogenized using a sterile pestle and total RNA was extracted using the Trizol kit (ABI). Extracts were treated with DNase, the concentration and purity of RNA determined on a Nanodrop 2000®, and 300 ng of total RNA was used for reverse transcription using the Phuson RT-PCR kit (Thermo Scientific). Primers for all RT-qPCR assays of *vitellogenin*, *defensin*, *cathepsinl* and the reference genes *Elongation factor alpha* and *18S* were designed using Primer3 and published sequences, or were directly taken from the literature when available [32]. All analyses were performed on an ABI OneStep qPCR machine using the OneStep analysing software.

5. Experiment 3—pathogen challenge

To determine the effect of treatment on the ability of queens to resist a pathogen, 72 nests (consisting of one queen, workers and brood) were randomly assigned to four different treatment groups. Two groups received a JHa treatment, whereas the other two groups were CoA control groups; all were treated over a period of two weeks. After two weeks, the queens were separated from their colony and individually placed in Petri dishes (90 mm diameter) containing moist cotton wool. Queens were challenged with 1 µl of *Metarhizium pingshaense* conidia suspended in 0.05% Triton-X (strain KVL02-73 [32,33]; 2.44×10^7 conidia ml^{-1}; conidia viability was more than 92%) applied topically, or treated with 1 µl of sterile 0.05% Triton-X control, and mortality was then recorded daily for 21 days [33,34]. The dose used of this pathogen kills around 25% of queens normally (see Results).

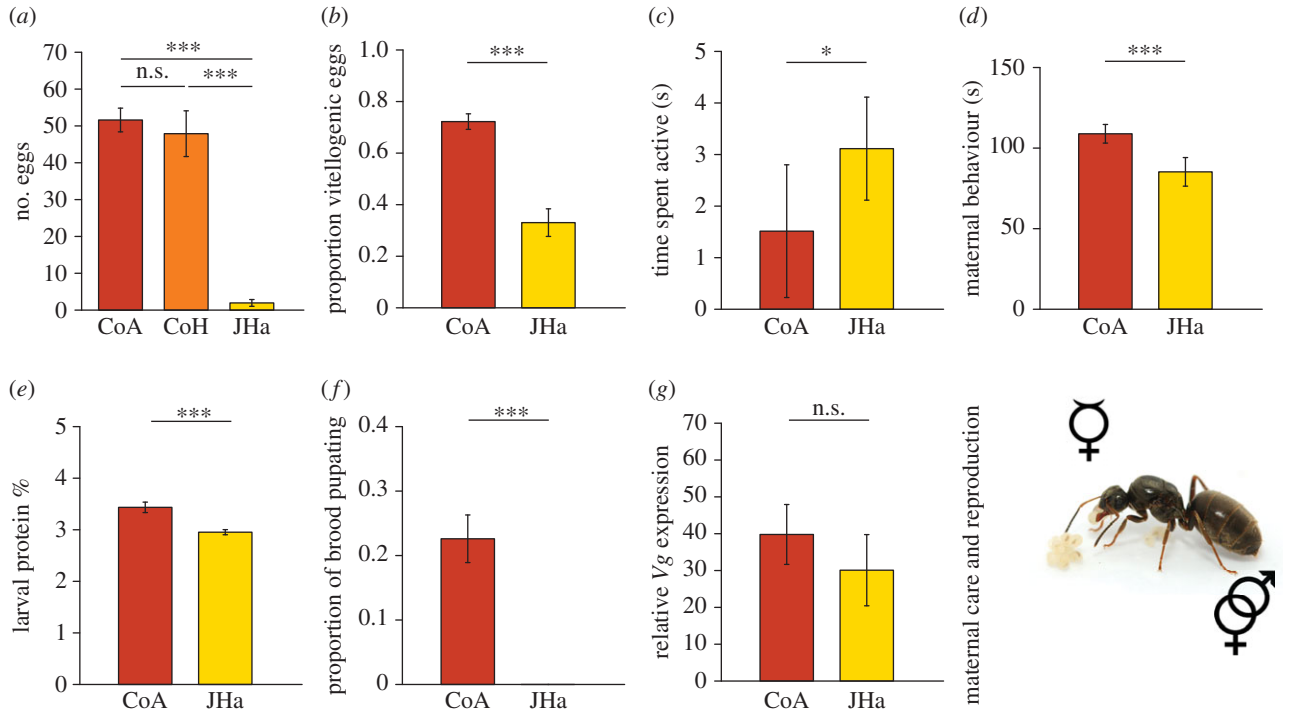

Figure 1. The mean ± s.e. effects of juvenile hormone on behavioural and reproductive traits of *Lasius niger* ant queens. (*a*) The number of eggs laid by queens in the different treatment groups during the two weeks of the experiment. (*b*) The proportion of eggs containing yolk (vitellogenic) versus eggs not containing yolk (non-vitellogenic) present in the ovaries of queens in the different treatment groups. (*c*) The time in seconds the queens spent being active during a 2 min observation. (*d*) The amount of time the queens spent performing maternal behaviour. (*e*) The amount of total protein present in larvae after three weeks of treatment (wet weight). (*f*) The proportion of pupae which had pupated at the end of the experiment (as a proxy for developmental speed). (*e*) The difference in relative *Vg* expression between different treatment groups in comparison to two reference genes (*18S* and *Elong1*). In all figures, CoA = acetone control, CoH = handling control (only present in figure 1*a*) and JHa = juvenile hormone analogue methoprene-treated queens. Significant differences between treatments are indicated by asterisks; 'n.s.' indicates they did not differ (error bars indicate ± s.e.). (Online version in colour.)

6. Data analysis

The data were non-normally distributed, so we used non-parametric tests to test for significant differences between treatment groups. We compared the egg laying rate of queens treated with JHa, solvent control or handling control in experiment 1 using a Kruskal–Wallis (KW) test. We compared the reproduction and immune variables between queens treated with JHa and solvent control in experiment 2 using Mann–Whitney–Wilcoxon (MWW) tests. In cases of multiple testing (KW), we used pairwise MWW as post hoc test, and the subsequent p values were corrected using the sequential Bonferroni method. Cox proportional hazard models, using the Breslow method were used to analyse queen survival in all three experiments with treatment as the predictor variable and queen longevity as the response variable. The model assumptions (i.e. proportional hazard) were tested before each analysis. In the case of more than two treatment groups (experiments 1 and 3), we used a pairwise Tukey post hoc test for group comparisons. The resulting p-values were corrected using the Holms method to account for multiple testing. All analyses were conducted in R 3.1.0 [35]. The package 'survival' [36] and the package 'multcomp' [37] were used for the survival analysis. The package 'sciplot' [38] was used for plot generation.

7. Results

Queens belonging to the different treatment groups did not differ in terms of size (all $p > 0.11$; overall 1.62 ± 0.058 mm head width) nor did hazard rates differ between the treatments during experiments 1 and 2 ($p > 0.075$ in all cases). Queens of all treatments had a similar number of brood or workers at the start of experiments 2 and 3 (all $p > 0.45$).

8. Reproduction and maternal care

The application of JHa had drastic effects on the reproductive physiology of *L. niger* queens. The egg-laying rate of young *L. niger* queens was significantly altered by JHa application ($n = 57$, KW $\chi^2 = 34.3$, d.f. = 2, $p < 0.001$; figure 1). JHa-treated queens laid fewer eggs compared with both the acetone and handling control (both Bonferroni adjusted $p < 0.001$; figure 1*a*). In addition, JHa application increased the proportion of non-vitellogenic eggs (eggs not containing yolk and which are not viable), compared with the control ($n = 39$, $W = 32$, $p < 0.001$; figure 1*b*). When looking at the behavioural effects of JHa applications, we found an overall increase in activity ($n = 70$, $W = 468.5$, $p = 0.022$, figure 1*c*) and a reduced investment in maternal care ($n = 70$, $W = 784$, $p = 0.009$; figure 1*d*). In addition, broods reared by JHa-treated queens exhibited a reduction in both protein content ($n = 54$, $W = 620.5$, $p < 0.0001$; figure 1*e*) and developmental speed ($n = 50$, $W = 537.5$, $p < 0.0001$; figure 1*f*). In contrast to these clear effects, gene expression analysis provided little support for a regulatory function of JHa in *Vg* expression in *L. niger* queens, with there being no significant effect of JHa ($n = 30$, $W = 149$, $p = 0.137$; figure 1*g*).

9. Immunity

JHa had pronounced, but pathway-specific effects on the regulation of the innate immune system in *L. niger*. There

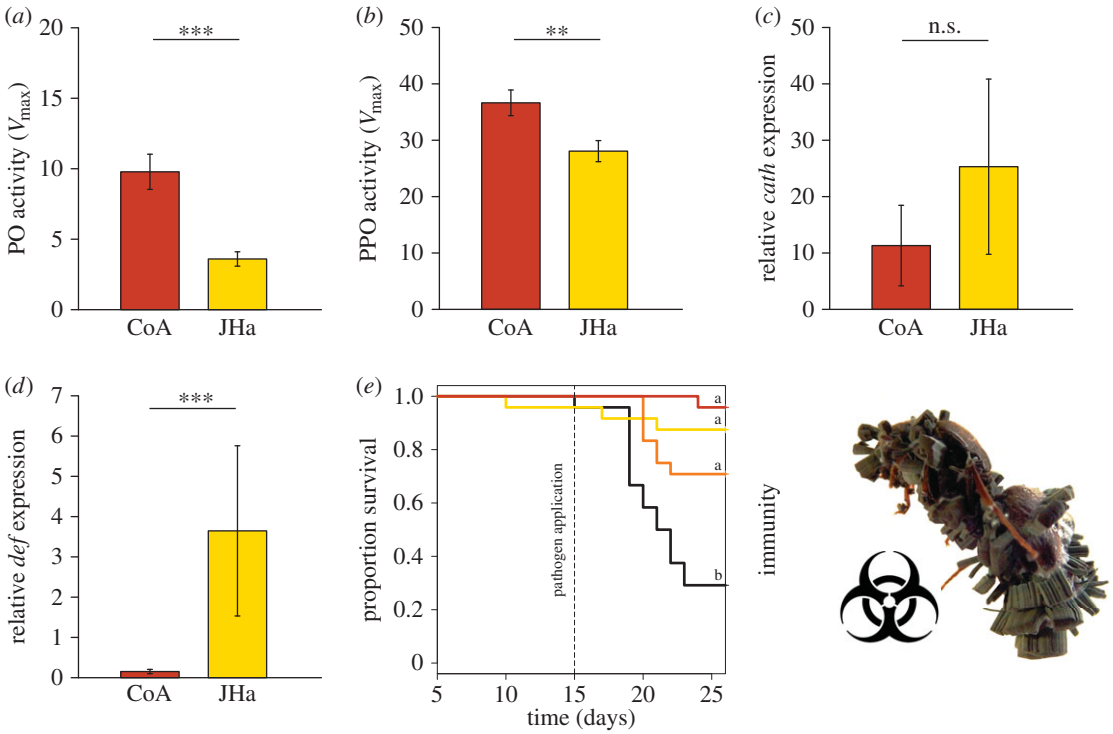

Figure 2. The regulatory effects of JH on the innate immune system of *Lasius niger* ant queens. (*a*,*b*) The effect of JH on the mean ± s.e. maximum activity of the phenoloxidase (PO) and pro-phenoloxidase (PPO) immune enzymes. Enzyme activity was measured during the linear phase of the reaction (V_{max}). (*c*,*d*) The mean ± s.e. relative expression of the *cathepsinI* and *defensin* immune genes, normalized against two reference genes (*18S* and *Elong1*). (*e*) The effect of JH on the resistance of queens to the fungal parasite *Metarhizium pingshaense* (black, JHa and parasite; yellow, JHa and control; orange, control and parasite; red, control and control). CoA, acetone control and JHa, juvenile hormone analogue methoprene-treated queens. Significant differences between treatments at $p < 0.05$ are indicated by asterisks in (*a*–*d*), and by different letters beside lines in (*e*). (Online version in colour.)

was a significant reduction in the activity of two important immune enzymes, PO and its precursor PPO in JHa-treated queens (PO: $n = 71$, $W = 1013.5$, $p < 0.0001$; figure 2*a*, and PPO: $n = 73$, $W = 818$, $p = 0.007$; figure 2*b*). In contrast JHa had no significant effect on *cath* expression ($n = 30$, $W = 89$, $p = 0.345$; figure 2*c*), and actually increased *def* expression ($n = 30$, $W = 14$, $p < 0.0001$; figure 2*d*). When queens were challenged with the fungal pathogen *Metarhizium pingshaense*, we found an overall effect of treatment on survival of parasite exposure ($n = 96$, $W = 21.7$, d.f. $= 3$, $p < 0.0001$), with JHa-treated queens exhibiting a significantly increased mortality rate compared with control queens when exposed to the parasite (figure 2*e*). The results of experiment 1–3 are summarized in figure 3.

10. Discussion

Our results indicate that queens of *L. niger* ants have escaped the RS trade-off by remodelling the endocrine regulation of reproduction. By releasing JH from its obligatory stimulating function during reproduction, *L. niger* queens are able to avoid extended periods of immune suppression. Given that pathogens (including fungal infections) are a leading cause of mortality in many insects in particular during the critical independent nest founding stage [24,39], the resulting increase in pathogen protection should translate into higher survival probability and consequently contribute to the queens' extraordinary lifespan. The striking reduction in reproductive output (egg laying rate) suggests that JH in *L. niger* queens has not only lost, but actually reversed its reproductive function, and now acts as a suppressor during the reproductive process. This situation superficially resembles the regulatory architecture of reproduction found in the honeybee *Apis mellifera*. Similar to our findings in *L. niger*, JH has reversed its gonadotropic function in *A. mellifera* queens [40,41] and now functions as a suppressor of *Vg* expression [42]. Given that we find that JH increases the production of non-vitellogenic eggs, but does not downregulate *Vg* expression directly, the inhibitory function of JH in *L. niger* must occur post-transcriptionally. Such post-transcriptional regulation could happen either directly at the protein level or by blocking the uptake of *Vg* into the developing oocytes. The latter seems the most likely possibility, because JH is known to regulate this process in other insect species, including social ones [9,40,41,43]. In contrast to the reversed role in regulating reproduction, the effects of JH on maternal behaviour exhibit a conserved pattern by suppressing maternal behaviour while increasing locomotor activity, similar to solitary insects [9,44]. The reduced investment in maternal behaviour could potentially explain the reduction in both protein content and brood developmental speed observed in JHa-treated queens. During the founding stage, queens of *L. niger* seal off the entrance to their new nest and rear the first generation of workers using their own energetic reserves [24]. If queens invest less time in maternal care, their offspring might suffer from malnutrition, which in turn could cause delay in worker maturation. This effect would likely result in severe fitness costs to young queens during the critical founding stage. An alternative explanation for our findings might be related to the well-documented role of JH in regulating moulting and caste determination in insects [45,46]. As JHa is likely transferred to the larvae, it might have inhibited the moulting process of late larvae and consequently prevented pupation.

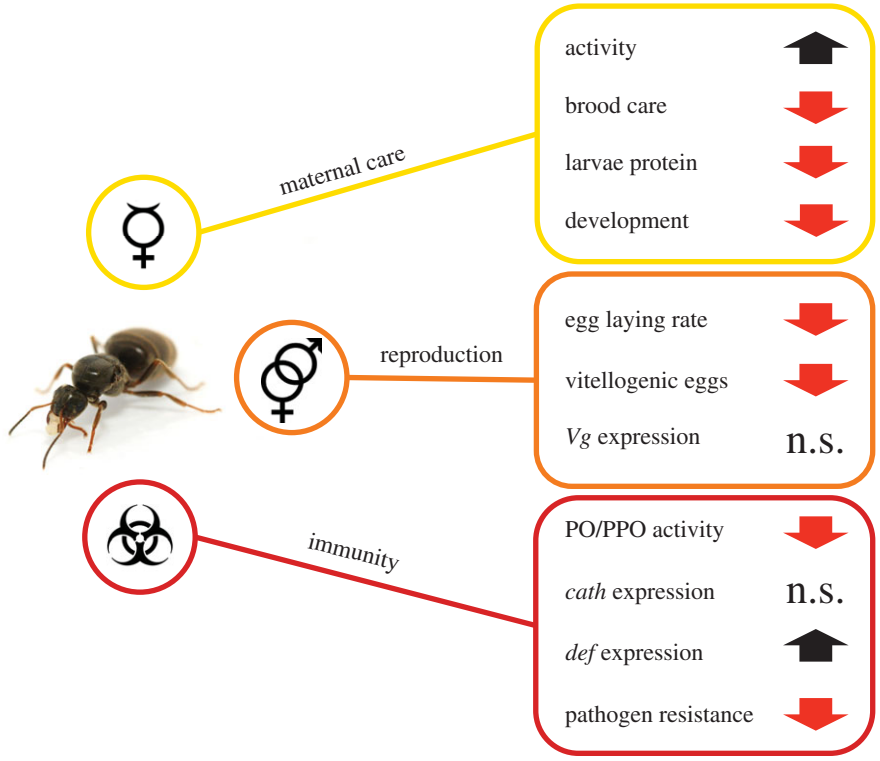

Figure 3. Summary of the effects of JHa on maternal behaviour, reproductive physiology and immunocompetence. A black arrow indicates a significant increase of the trait in question in response to JHa treatment, whereas a red arrow indicates a significant decrease of the trait in response to JHa. 'n.s.' indicates that JHa treatment did not have a significant effect. (Online version in colour.)

Compared with the relatively straightforward role of JH in both reproduction and maternal behaviour, its role in regulating the innate immune system appears more complex. We investigated the effect of JH on three key pathways of the innate immune system. We find that, similar to most systems studied so far, JH acts as a potent suppressor of the PO/PPO pathway [12,13,15], yet in contrast we find no effect on the expression of *cath* and a stimulatory effect on the expression of *def*. A lack of information regarding the regulatory role of JH in the innate immune system hinders a meaningful comparison with the only other ant species in which the role of JH in reproduction has been thoroughly investigated. In queens of the red imported fire ant *Solenopsis invicta*, JH still acts as a gonadotropin stimulating *Vg* expression [40,41,43], but the role of JH in the innate immune system is unknown. *S. invicta* might have escaped the RS trade-off using the alternative pathway by decoupling the regulatory effects of JH on the innate immune system instead of changing its regulatory role in reproduction. In a recent study, Libbrecht *et al.* [46] document a similar function of JH of in queens of *Pogonomyrmex rugosus*. These results suggest that JH's function is not conserved in ants and can act as a flexible tool in regulating key systemic processes in different genera.

When looking at a broader phylogenetic scale, we find indirect support for the presence of a JH-mediated RS trade-off. In the ant genus *Diacamma*, reproduction is associated with both a low JH titre and an increased lifespan [47,48]. In contrast to these findings, all studies support the role of JH as a gonadotropin in bumblebees, wasps and primitively eusocial bees, and high reproductive output is associated with elevated JH titres [40,41]. These are all species in which queens have relatively short lifespans, similar to those of solitary insects.

The antagonistic role that we find in *L. niger* of JH in stimulating *def* expression while suppressing the PO/PPO pathway is somewhat surprising. The three immune pathways are involved in fighting off different types of pathogens: the PO/PPO pathway is involved in fungal defence and wound healing [32,49], whereas *def* and *cath* are key mediators in the immune response raised against bacterial and viral infections, respectively [50–55]. It has been recently demonstrated that mating is an essential component in triggering an additional level of insect immunity. Galvez & Chapuisat [56] show that mated queens of *L. niger* exhibit increased resistance against the fungal pathogen *Beauveria bassiana*. Our findings could provide a functional mechanism for their findings, because the reproductive period in *L. niger* queens is likely characterized by a low JH titre and this endocrinological state promotes fungal resistance. The increased susceptibility of unmated *L. niger* queens might indicate that maturing queens, similar to *S. invicta* queens [57], might require elevated JH titres and could pay they costs of the associated immunosuppression.

In this context, we would like to discuss the potential interaction of JH and a second major endocrinological regulator ecdysone (Ecd). During development JH and Ecd interact to orchestrate the moulting process, while regulating both the reproductive cycle and insect innate immune system in adults of some insects [45,58,59]. Their often-complex interactions [60] make them promising targets to investigate their role in regulating some key systemic processes in *L. niger* queens. While Ecd seem to have lost its reproductive function in some social insect linages [60], its regulatory role in the immune system remains to be investigated.

This study demonstrates that the antagonistic effects underlying endocrine-mediated, as well as energetic trade-offs, can and will be decoupled given the right selective environment [61]. In the case of social insects, selection has strongly favoured the evolution of both a long lifespan and high reproductive

output. The theory of ageing predicts that long lifespans should only be selected for if extrinsic mortality is low, a condition met in the queens of many eusocial insects [62,63]. Once queens have survived the hazardous colony-founding stage, they live in an extremely sheltered environment deep within the colony. At the same time, reproductive division of labour, which is characteristic of all eusocial insects, removes potential nutritional and behavioural (maternal care) limitations on reproductive output because these tasks are outsourced to sterile helpers [64]. Under these rare conditions, selection will strongly favour the decoupling of all trade-off-mediated constraints and select for both increased lifespan and high reproductive output, thus abolishing the costs of reproduction. This suggests that energetic and physiological trade-offs do not ultimately inhibit the evolution of extreme life-history strategies. Consequently, the scarcity of reproductive phenotypes maximizing reproduction and longevity simultaneously likely reflects a lack of selective pressure to overcome trade-offs, rather than trade-off-mediated constraints on evolutionary potential.

Data accessibility. All data are available in Dryad at http://dx.doi.org/10.5061/dryad.3rq87.

Authors' contributions. T.P. and W.O.H.H. designed the experiment, T.P. and D.T. carried out the experiment, T.P. analysed the data and wrote the first draft of the manuscript. T.P., D.T. and W.O.H.H. wrote the final version of the manuscript.

Competing interests. We declare we have no competing interests.

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