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RESEARCH ARTICLE

Juvenile hormone downregulates vitellogenin production in *Ectatomma tuberculatum* (Hymenoptera: Formicidae) sterile workers

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ABSTRACT

In the ant *Ectatomma tuberculatum* (Olivier 1792), workers have active ovaries and lay trophic eggs that are eaten by the queen and larvae. Vitellogenins are the main proteins found in the eggs of insects and are the source of nutrients for the embryo in the fertilized eggs and for adults in the trophic eggs. In social insects, vitellogenin titres vary between castes and affect reproductive social status, nursing, foraging, longevity, somatic maintenance, and immunity. In most insects, vitellogenin synthesis is mainly regulated by juvenile hormone. However, in non-reproductive worker ants, this relationship is poorly characterized. This study determined the effects of juvenile hormone on vitellogenin synthesis in non-reproductive *E. tuberculatum* workers. Juvenile hormone was topically applied onto workers, and the effect on vitellogenin synthesis in the fat body and vitellogenin titres in the haemolymph were analysed by ELISA and qPCR. Juvenile hormone downregulated protein synthesis and reduced vitellogenin titres in the haemolymph, suggesting that in workers of *E. tuberculatum*, juvenile hormone loses its gonadotrophic function.

KEY WORDS: Vitellin, Age polyethism, Ant, Hymenoptera

INTRODUCTION

Insect vitellogenins are proteins synthesized mainly in the female fat body and released into the haemolymph (Tufail and Takeda, 2008; Azevedo et al., 2011), and are later transferred to oocytes via receptor-mediated endocytosis (Tufail and Takeda, 2009) and stored as vitellins in yolk granules (Raikhel and Dhadialla, 1992; Giorgi et al., 1999). The vitellins are the major egg yolk proteins in insects and are important for embryo nourishment (Raikhel and Dhadialla, 1992; Giorgi et al., 1999; Tufail and Takeda, 2008). However, in social insects such as eusocial Hymenoptera, vitellogenins have physiological functions different from embryo nutrition, in which sterile bee and ant workers also produce vitellogenin (Excels, 1974; Gobin et al., 1998; Wheeler et al., 1999; Dallacqua et al., 2007).

In the honeybee *Apis mellifera*, vitellogenins are multifunctional proteins, playing a role in brood feeding and royal jelly production (Amdam et al., 2003), age polyethism regulation (Antonio et al., 2008), antioxidation (Seehuus et al., 2006), immunity regulation (Amdam et al., 2004; Seehuus et al., 2006), juvenile hormone (JH) synthesis (Guidugli et al., 2005) and insulin/insulin-like signaling, which controls growth, aging and reproduction (Corona et al., 2007). In worker ants, vitellogenin is associated with age polyethism (Fénéron et al., 1996; Azevedo et al., 2011), the nourishment of larvae and adults by the production of trophic eggs (Camargo Mathias and Caetano, 1995; Fénéron and Billen, 1996; Peeters, 1997; Gobin et al., 1998; Dietemann and Peeters, 2000; Khila and Abouheif, 2008) and polyphenism (Libbrecht et al., 2013).

Vitellogenin dynamics in adult insects is closely related to JH (Wyatt et al., 1996). The synthesis and uptake of vitellogenin by oocytes are controlled by JH (Robinson and Vargo, 1997; Hartfelder, 2000), and vitellogenin and JH both coordinate the caste determination as well as labor division in workers of social Hymenoptera (Guidugli et al., 2005). In species with low morphological differentiation between queens and workers such as the bumble bee *Bombus terrestris*, the wasps *Polistes canadensis* and *Polistes gallicus*, and the bee *Lasioglossum zephyrum*, in which the workers can reproduce in the absence of a queen, the JH levels are positively related to vitellogenin titre and ovary activation (Bell, 1973; Röseler et al., 1984; Bloch et al., 2000; Giray et al., 2005). In contrast, JH does not act as a gonadotropin in all species of bees and ants. In *A. mellifera* adults, the production of vitellogenin is downregulated by high JH titres in haemolymph (Pinto et al., 2000). The positive effect of JH on vitellogenin synthesis in *A. mellifera* occurs only during the pupal stage (Barchuk et al., 2002). In the ants *Lasius niger* and *Monomorium pharaonis*, topical application of JH in queens decreases egg production and ovary size (Sommer and Holldobler, 1995; Hsieh and Su, 2000). In several species of ants lacking a morphologically differentiated queen caste, such as *Diacamma* sp. and *Streblognathus peetersi*, the active reproductive workers (gamergates) have lower levels of circulating JH than the subordinate non-reproductive workers (Sommer et al., 1993; Brent et al., 2006). Thus, in ants, the roles of JH hormone in vitellogenin regulation have been studied mainly in queens and gamergates. Recent molecular studies have shown that the vitellogenin gene experienced duplication in several ant genomes and the expression rates of the different copies differs between queens and workers (Corona et al., 2013; Morandin et al., 2014), but, to date, data on hormonal coordination of vitellogenin production in ants are scarce, and species in which workers lay eggs in queenright colonies are a good model with which to test the non-gonadotropic effect of JH.

Ants belonging to Ectatomminae have basal morphological and social biology traits, such as predatory habits, limited queen–worker dimorphism, spermatheca retention in workers, semi-claustral pattern of colony foundation, and absence of worker dimorphism

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(Peeters, 1997). *Ectatomma tuberculatum* (Olivier 1792) (Ectatomminae) makes small colonies of up to 400 workers with one or many morphologically differentiated queens (Hora et al., 2001; Hora et al., 2005). The workers are morphologically identical, and the tasks they perform within the colony vary according to age (Champalbert and Lachaud, 1990; Fénéron et al., 1996; Hora et al., 2001). The workers of *E. tuberculatum* also have active ovaries and produce non-reproductive trophic eggs (Fénéron and Billen, 1996; Hora et al., 2007). Furthermore, they are not able to mate because their spermathecae are not functional (Hora et al., 2001). In this ant, vitellogenin is associated with age polyethism in workers (Azevedo et al., 2011).

Studies of vitellogenin regulation by JH in ants are mainly based on queens and on species in which workers have the potential to become queens (Sommer et al., 1993; Sommer and Holldobler, 1995; Burns et al., 2002; Brent et al., 2006). The present study was conducted to obtain information about the relationship between JH and vitellogenin synthesis in non-reproductive ant workers with active ovaries, and to test whether JH downregulates the vitellogenin production in *E. tuberculatum* adult workers.

MATERIALS AND METHODS

Insects

Six colonies of *E. tuberculatum* were collected at the Cocoa Research Center, Itabuna, BA, Brazil, and transferred to the Laboratory of Cell Ultrastructure of the Department of General Biology at the Federal University of Viçosa, Viçosa, MG, Brazil, where they were reared in artificial colonies in plastic cages (18×25 cm) filled with plaster and connected by a tube to another plastic compartment (10×10 cm) used as a foraging arena. All colonies were polygynous, containing from two to five queens and more than 30 workers and brood. The colonies were kept at 26±2°C and fed every 2 days with larvae of *Tenebrio molitor* Linneaus 1758 (Coleoptera: Tenebrionidae), honey, and water *ad libitum* (Hora et al., 2007).

To obtain ants of known ages, newly emerged workers were marked with an enamel paint dot on the thorax and kept in the artificial nest of origin until use.

Antibody production

Antibodies against *E. tuberculatum* vitellogenin were obtained by the immunization of rabbits with the 156 kDa vitellin from physogastric queen and worker eggs as described by Azevedo et al. (2011). BALB/c mice up to 3 weeks old were also immunized with this vittelin from worker eggs to be used in ELISA assays. Three immunizations were performed, the first using a total of 0.25 mg of proteins with Freund's complete adjuvant (v/v). The second and third booster immunizations were performed 30 and 60 days after the first, respectively, using a total of 0.05 mg of proteins in incomplete Freund's adjuvant (v/v) each. Blood was collected 30 days after the third immunization and the serum obtained was stored at −20°C.

Western blotting

The anti-vitellogenin antibodies were initially tested to detect vitellins in *E. tuberculatum* worker egg, haemolymph and fat body extracts by western blotting. Adult workers 30 days old were dissected and the mature eggs, haemolymph and fat body were obtained and homogenized in protein extraction buffer [0.1 mol l^{-1} phosphate-buffered saline (PBS), pH 7.2, 0.1 mol l^{-1} NaCl, 2.5 mmol l^{-1} Na_2EDTA, 0.5% (v/v) Triton X-100, 5% (v/v) glycerol and 10% (v/v) protease inhibitor cocktail-4-(2-aminoethyl) benzenosulfonil fluoride (AEBSF), E-64, bestatina, leupeptina, aprotinin and sodium EDTA; Sigma], followed by centrifugation and measurement of total protein content using the bicinchoninic acid (BCA) method (Smith et al., 1985). Extracts containing 20 µg of total protein were subjected to 10% SDS-PAGE (Laemmli, 1970), followed by transfer of the proteins to 0.2 µm pore size nitrocellulose membranes (Sigma-Aldrich, catalog no. N7892-5EA). The membranes were blocked with 5% non-fat dried milk in 0.1 mol l^{-1} TBS buffer, pH 8.0, containing 0.1% Tween-20 (TBST), and posteriorly incubated separately for 2 h at room temperature with the rabbit and mouse immune-serum diluted 1:1000 in TBST, followed by incubation with anti-rabbit and anti-mouse IgG conjugated to horseradish peroxidase (Sigma) diluted 1:4000 in TBST, both solutions supplied with 2.5% non-fat dried milk. The negative control was performed by replacing the immune serum by mouse pre-immune serum diluted 1% in TBST. The membrane was revealed with DAB/H_2O_2 solution (0.1% 3-3′-diaminobenzidine in 50 mmol l^{-1} Tris-HCl, pH 7.6, 2.5% of 0.3% nickel chloride in H_2O, 0.1% H_2O_2).

JH treatment

The JH treatment was performed with vitellogenin-producing workers of *E. tuberculatum* aged between 20 and 30 days (Azevedo et al., 2011). Vitellogenin production was confirmed by verifying the presence of mature oocytes in the ovaries after the collection of the haemolymph and fat body.

The workers received a topical application onto the abdomen of 0.2, 1 and 5 µg of JH III (Sigma) diluted in pure acetone (Merck). The no-treatment control group received the application of the same volume (3 µl) of water, and a second control group received the application of the same volume (3 µl) of pure acetone. Workers were randomly collected from the six colonies, and after treatment they were placed individually into plastic cages supplied with water and honey for 16 h; the haemolymph and fat body were collected after this period. Over 15 workers that received the highest JH dose were marked and returned to their colony of origin, where they were observed for at least 10 days. During this time, these workers showed 86% of survival.

Haemolymph and fat body sample preparation

For haemolymph sampling, 45 workers were anesthetized at 0°C and decapitated, and 0.5 and 0.75 µl of haemolymph from each ant were collected with the aid of a microcapillary tube and transferred to 25 µl of protein extraction buffer as previously described. The samples were centrifuged at 9000 ***g*** for 10 min, followed by the collection of the supernatant and measurement of total protein content using the BCA method. Five samples of 25 µl solution containing pooled haemolymph samples of three ants were made for each treatment and/or period.

To determine the level of vitellogenin in the fat body, 45 workers were anesthetized at 0°C, and the fat body was dissected and transferred to 200 µl of extraction buffer as previously described. Five samples of 200 µl solution containing fat body of three ants were prepared for each treatment/period. The samples were macerated and centrifuged at 9000 ***g*** for 10 min, followed by the collection of the supernatant and measurement of total protein using the BCA method.

ELISA

The level of vitellogenin in the fat body and haemolymph was measured by ELISA in 96-well plates. The wells were sensitized with rabbit anti-vitellogenin antibody diluted 1:5000 in 0.05 mol l^{-1} carbonate/bicarbonate buffer, pH 9.6, for 16 h at 4°C, and blocked with 1% bovine serum albumin in 0.1 mol l^{-1} PBS, pH 8.0, plus 0.05% v/v Tween (PBST) for 2 h at room temperature. After washing with PBST, the wells were filled with samples of haemolymph and fat body diluted in PBST in a final volume of 100 µl for 2 h at room temperature. For haemolymph analyses, 5 µg of total protein was applied to the wells, whereas the wells for fat body extract used 20 µg of total protein. After further washing in PBST, the samples were incubated for 2 h at room temperature with mouse anti-vitellogenin antibody diluted 1:1000 in PBST, followed by washing and incubation with anti-mouse IgG produced in rabbit conjugated with horseradish peroxidase (Sigma) diluted 1:9000 in PBST for 2 h at room temperature. Visualization was achieved using 3,3′,5,5′-tetramethylbenzidine (TMB) solution (Sigma) in 0.05 mol l^{-1} phosphate/citrate buffer, pH 5.0, according to the manufacturer's instructions. The reaction was stopped with 20 µl of 5% H_2SO_4 solution and analysed in a spectrophotometer at 450 nm.

The amount of vitellogenin in the samples of haemolymph and fat body was calculated based on the equation obtained from the standard curve that used different dilutions of antigen from *E. tuberculatum* worker egg extracts

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Table 1. Primers used in the real-time qPCR analyses (5′–3′ order)

Gene	Forward	Reverse
Vitellogenin	AGAATTTGCCGCTACCTACGCT	CATTTCTGAGCTTGAATTGCAGGA
β-Actin	TGCCAACACTGTCCTTTCTG	AGAATTGACCCACCAATCCA

with known protein concentrations. Samples of haemolymph and fat body were measured in triplicate.

Quantitative real-time PCR

The effect of JH on *vitellogenin* gene expression was analysed by quantitative real-time PCR (qPCR), comparing the expression of vitellogenin mRNA in 20- to 30-day-old workers treated with 5 µg JH III or pure acetone, as described previously.

The treated ants (seven for each treatment) were macerated in liquid nitrogen 16 h after treatment, and total RNA was extracted with Tri-Reagent (Sigma) according to the manufacturer's instructions. Total RNA was quantified in a spectrophotometer at 260 nm, and its quality was determined using the ratio between the absorbance measured at 260 and 280 nm. The single-strand cDNA was made in 20 µl reactions containing 2 µg of total RNA, 40 µmol l^{-1} oligo-$(dT)_{20}$ primers (Invitrogen), 10 mmol l^{-1} dNTP mix (Invitrogen), 40 U Murine RNAse inhibitor (New England BioLabs) and 200 U M-MuLV Reverse Transcriptase (New England BioLabs) diluted in buffer provided by the enzyme kit manufacturer. The reactions were incubated at 42°C for 1 h, and the transcriptase was inactivated at 80°C for 5 min.

The qPCR reaction was performed using a 7500 Real-Time PCR System (Applied Biosystems) thermocycler using the fluorescent quantification kit GoTaq qPCR Master Mix (Promega). Paralog-specific primers for *vitellogenin1* (GenBank CB252008; Tian et al., 2004), and reference gene *actin* (GenBank AB023025; Dallacqua et al., 2007) (Table 1) were designed using sequence alignment (Thompson et al., 1997) and primer analysis (Rychlik, 2007) programs. Each cDNA sample was analysed in 50 µl reactions assembled in duplicate using 2× GoTaq qPCR Master Mix, 400 nmol l^{-1} forward and reverse primers, 100 ng of cDNA and ultra-pure water supplied with the qPCR kit. The PCR reaction involved initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and elongation at 72°C for 2 min. The quantification and calculation of *vitellogenin* expression were performed using the ΔΔCT method (Pfaffl et al., 2002).

Statistics

The amounts of vitellogenin per microgram of total protein in the haemolymph and fat body data for the controls (water and acetone) and the three different JH doses showed a normal distribution and were compared using one-way ANOVA followed by Dunnett's multiple comparison post-test at a 5% significance level. The gene expression data were fit using arcsin transformation to reach a normal distribution followed by a *post hoc* unpaired *t*-test at a 5% significance level.

RESULTS

The 156 kDa vitellogenins present in *E. tuberculatum* worker egg, haemolymph and fat body (Fig. 1A) were detected by the anti-vitellogenin polyclonal antibodies produced in rabbit (Fig. 1B) and mouse (Fig. 1C). These antibodies were used in ELISA essays to measure whether JH affects the amount of circulating vitellogenin in the haemolymph and its level in the fat body.

The levels of circulating vitellogenin in the haemolymph were influenced by JH (ANOVA, $F_{4,21}$=7.919, P<0.001; Fig. 2). The vitellogenin amounts (means±s.e.m.) were similar among water [0.31±0.07 µg vitellogenin(VG) μg^{-1} total proteins(TP)] and acetone (0.30±0.09 µg VG μg^{-1} TP) controls and 0.2 (0.22±0.03 µg VG μg^{-1} TP) and 1.0 µg JH treatments (0.21±0.01 µg VG μg^{-1} TP; Fig. 2). Only workers treated with 5 µg JH had a significant decrease in circulating vitellogenin titres (0.11±0.03 µg VG μg^{-1} TP) when compared with controls (Fig. 2).

To test the hypothesis that vitellogenin depletion in haemolymph may be linked to increasing protein uptake by eggs in ants treated with JH, we verified the effect of JH in vitellogenin synthesis by the fat body. At first, we measured the amount of vitellogenin in the fat body. The JH treatment reduced vitellogenin protein levels in the fat body of treated *E. tuberculatum* workers (ANOVA, $F_{4,18}$=9.458, P<0.0001; Fig. 3). The acetone treatment did not affect the protein levels in the fat body compared with the water control, with 22.08±11.0 and 22.12±12.72 ng VG μg^{-1} TP, respectively (Fig. 3). Ants treated with the three JH doses showed a decrease in fat body vitellogenin levels compared with controls, with 1.27±0.82, 2.16±1.12 and 1.26±0.45 ng VG μg^{-1} TP for treatments with 0.2, 1 and 5 µg of JH, respectively (Fig. 3).

To confirm the link between the JH-induced reduction of vitellogenin levels in the fat body and haemolymph, we also performed a quantitative real-time PCR (qPCR) analysis of a *vitellogenin* gene in workers of *E. tuberculatum* treated with JH. For this, we used a 5 µg dilution of JH, which decreased vitellogenin in both haemolymph and fat body. The JH treatment downregulates *vitellogenin* expression in relation to control ants (t=2.91, d.f.=14, P=0.007; Fig. 4).

DISCUSSION

Our results show that the levels of vitellogenin decrease in the fat body and haemolymph of adult sterile *E. tuberculatum* workers after hormone application, indicating that the production of this protein is downregulated by an increase in circulating JH. The use of acetone as JH solvent can affect the morphology of fat body cells (Paes-de-Oliveira et al., 2008). Therefore, we set up two control treatments consisting of topical aplication of pure acetone and water to verify

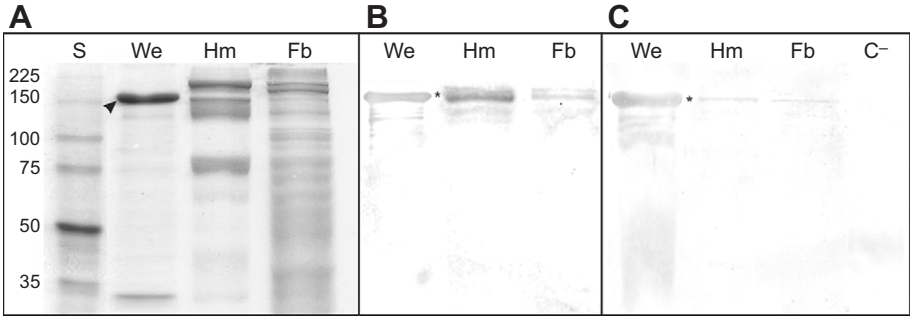

Fig. 1. Vitellogenin in *Ectatomma tuberculatum*. (A) Protein profile of worker egg (We), haemolymph (Hm) and fat body (Fb) obtained by SDS-PAGE. The arrowhead indicates the 156 kDa vitellogenin. S: molecular weight standard proteins followed by respective values in kDa (Promega Broad Range Protein MW Marker). (B) Western blotting showing the detection (*) of 156 kDa vitellogenin in extracts of eggs, haemolymph and fat body using antibodies produced in rabbits. (C) Detection of the 156 kDa vitellogenin (*) in extracts of eggs, haemolymph and fat body using antibodies produced in mouse. C–: negative control.

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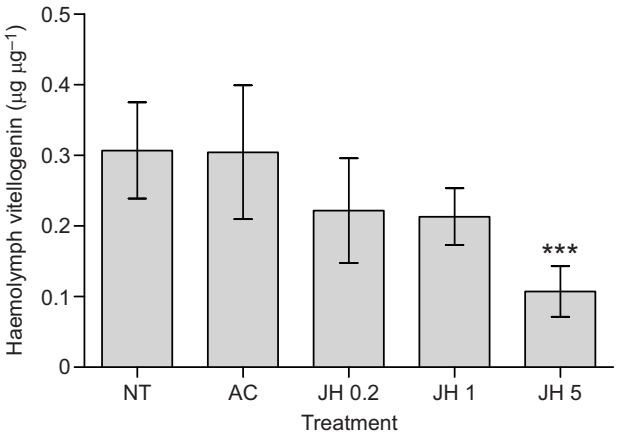

Fig. 2. Vitellogenin levels in the haemolymph of *Ectatomma tuberculatum* workers. Mean (±s.e.m.) vitellogenin levels (μg vitellogenin μg^{-1} total protein) in haemolymph samples of workers treated with water (NT), pure acetone (AC) and 0.2, 1 and 5 μg of juvenile hormone (JH) diluted in acetone. Asterisks indicate a significant difference relative to controls based on ANOVA followed by Dunnett's multiple comparison test, ***P<0.001.

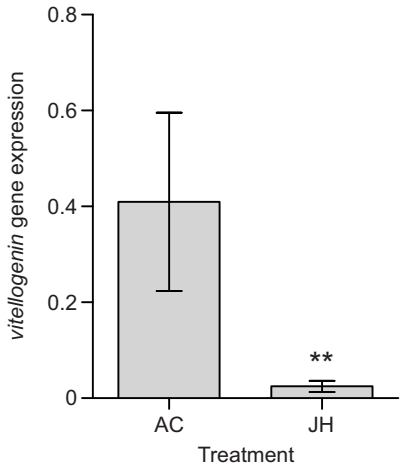

Fig. 4. *Vitellogenin* mRNA expression in *Ectatomma tuberculatum* workers. Mean (±s.e.m.) *vitellogenin* (*vg*) mRNA expression in relation to *β-actin* mRNA expression in workers treated with pure acetone (AC) and 5 μg of JH III (JH) diluted in acetone. Asterisks indicates a significant difference based on unpaired *t*-tests, **P=0.007.

whether the acetone can interfere in vitellogenin production. The vitellogenin levels were similar in acetone- and water-treated ants, showing that it was affected by JH and not by the acetone used as hormone solvent.

A gonadotropic role for JH in ants has been demonstrated in queens of *Solenopsis invicta*, related to the synthesis of vitellogenin receptors and its uptake by oocytes (Lewis et al., 2001; Burns et al., 2002; Brent and Vargo, 2003; Chen et al., 2004). In *S. invicta*, there is a peak of JH in the haemolymph of queens during the oviposition period (Brent and Vargo, 2003). JH activates ion channels in ovarian follicular cells, increasing the intercellular space as a result of changes in the architecture of the actin filaments (Ronnau et al., 2015) and facilitating vitellogenin to reach the surface of oocytes, where it is endocytosed (Dhadialla et al., 1992; Tufail and Takeda, 2009). In this way, the reduction of the circulating vitellogenin in the haemolymph in JH-treated ants may be associated with the uptake of vitellogenin for growing oocytes, since all workers used in the experiment have developing trophic eggs (Hora et al., 2001, 2007). On the contrary, in *S. invicta*, the JH peak occurs in queens during the oviposition period (Brent and Vargo, 2003), but in this fire ant, worker are always sterile. Further, our data showed that the depletion of vitellogenin in the haemolymph of *E. tuberculatum* workers exposed to JH was also associated with the downregulation of *vitellogenin* gene expression, evidenced by qPCR and the consequent reduction of vitellogenin protein synthesis in the fat body showed by ELISA. The different response of the fat body and haemolymph linked to the JH dose may be due to the delay between gene transcription in the fat body and protein release to the haemolymph, as reported in other insects (Glinka and Wyatt, 1996). Furthermore, changes in the circulating vitellogenin in the haemolymph after topical application of JH has been reported to be dose responsive (Davis et al., 1990; Hiremath and Jones, 1992).

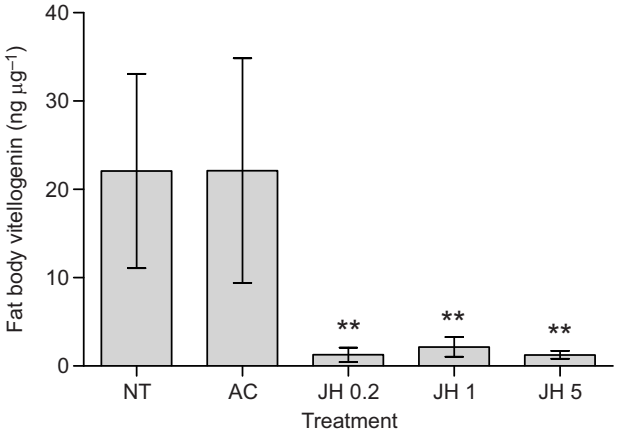

Fig. 3. Vitellogenin levels in the fat body of *Ectatomma tuberculatum* workers. Mean (±s.e.m.) vitellogenin levels (ng vitellogenin μg^{-1} total protein) in the fat body of workers treated with water (NT), pure acetone (AC) and 0.2, 1 and 5 μg of juvenile hormone (JH) diluted in acetone. Asterisks indicate a significant difference based on ANOVA followed by Dunnett's multiple comparison test, **P<0.0001.

In most adult insects, JH stimulates vitellogenin synthesis (Hagedorn and Kunkel, 1979), as in some primitively eusocial Hymenoptera (Bell, 1973; Röseler et al., 1984; Bloch et al., 2000; Giray et al., 2005). However, vitellogenin synthesis has an inverse relationship with JH titres in adults of some highly eusocial Hymenoptera (Robinson and Vargo, 1997; Hartfelder, 2000). In adult workers of *A. mellifera*, an increase in JH titre in the haemolymph under natural conditions is related to lower haemolymph vitellogenin titres (Excels, 1974; Fluri et al., 1982), and topical application of the JH analog pyriproxyfen in workers and queens inhibits vitellogenin synthesis (Pinto et al., 2000). In the Ponerinae ant *Streblognathus peetersi*, the alpha reproductive worker is the only individual in the nest that has developed ovaries and high titres of vitellogenin in the haemolymph, but treatment with JH induces a decrease in the levels of circulating vitellogenin, and affects their reproductive status (Cuvillier-Hot et al., 2004). Our data indicate that *E. tuberculatum* follows the pattern observed in the honeybee and *S. peetersi*, suggesting that in some highly eusocial Hymenoptera, JH loses its vitellogenic function in adults. However, this is not true for all highly eusocial Hymenoptera, because the JH analog metoprene stimulates *vitellogenin* expression in *Pogonomyrmex rugosus* queens (Libbrecht et al., 2013).

Changes in the putative physiological function of vitellogenin in ants may be linked to duplication of *vitellogenin* genes (Corona et al., 2013). In some ant species with duplicated *vitellogenin* genes,

it has been reported that the differential gene expression in workers is correlated with the production of reproductive (Morandin et al., 2014) and trophic eggs (Corona et al., 2013) as well as with labor division (Wurm et al., 2011), showing that vitellogenin coded by different genes has subfunctions such as embryo nutrition (functional eggs), feeding the queen (trophic eggs) and playing a role in age polyethism (sterile workers). The genome of *E. tuberculatum* has not yet been sequenced, so we are not able to determine the number of *vitellogenin* copies. However, vitellogenin in *E. tuberculatum* workers is involved in age polyethism (Azevedo et al., 2011), and our findings suggest that JH is part of the regulatory pathway. Because in the studies mentioned above, the effects of JH on *vitellogenin* expression patterns are unknown, our findings opened a new avenue to test whether JH has different regulatory pathway in copies of these genes.

The honeybee *A. mellifera* is the main insect model for studying the effects of vitellogenin in age polyethism in social Hymenoptera (Amdam et al., 2009). In this species, young workers remain within the colony, executing tasks associated with brood care, whereas older workers are foragers (Michener, 1974). Vitellogenin appears as the main protein in the haemolymph of *A. mellifera* nurse workers (Pinto et al., 2000), and reaches its lowest amount in foragers (Excels, 1974; Nelson et al., 2007). Similarly, newly emerged workers of *E. tuberculatum* have low levels of vitellogenin in the haemolymph, which gradually increase to higher levels in the workers that perform nursing activities, whereas in foragers, vitellogenin synthesis is very low or even absent (Azevedo et al., 2011). A similar pattern was observed in sterile workers of the ant *Pogonomyrmex barbatus*, where the vitellogenin gene *Pb_Vg1* is more highly expressed in nurses than foragers (Corona et al., 2013). In *A. mellifera*, the decrease in the amount of vitellogenin in the haemolymph of foraging workers is linked to an increase in circulating JH levels associated with age (Fluri et al., 1982; Huang et al., 1991; Jassim et al., 2000). Our findings suggest that the levels of JH and vitellogenin are also inversely related in adult *E. tuberculatum* workers, indicating that *P. barbatus* and the bee *A. mellifera* share a similar physiological route controlling vitellogenin levels, and that age polyethism in the sterile workers of ants and bees may be determined by the same mechanisms.

In conclusion, the synthesis of vitellogenin in the fat body and the amount of circulating vitellogenin in the haemolymph of *E. tuberculatum* workers decreases under the influence of an input of JH, showing that this hormone has a negative effect circulating levels of vitellogenin in sterile workers of highly eusocial Hymenoptera that already synthesize vitellogenin.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
D.O.A. and J.E.S. designed the experiments; D.O.A. collected ants and performed the experiments; D.O.A. and S.O.d.P. analysed molecular data; D.O.A. and J.E.S. analysed protein data; D.O.A. and L.C.M. performed statistical analyses; D.O.A., J.C.Z. and J.E.S. wrote the paper; and all authors contributed to the final version of the manuscript.

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References
Amdam, G. V., Norberg, K., Hagen, A. and Omholt, S. W. (2003). Social exploitation of vitellogenin. *Proc. Natl. Acad. Sci. USA* **100**, 1799-1802.
Amdam, G. V., Simões, Z. L. P., Hagen, A., Norberg, K., Schrøder, K., Mikkelsen, Ø., Kirkwood, T. B. and Omholt, S. W. (2004). Hormonal control of the yolk precursor vitellogenin regulates immune function and longevity in honeybees. *Exp. Gerontol.* **39**, 767-773.
Amdam, G. V., Ihle, K. E. and Page, R. E., Jr (2009). Regulation of honeybee worker (*Apis mellifera*) life histories by vitellogenin. In *Hormones, Brain and Behavior*, 2nd edn (ed. W. P. Donald, P. A. Arthur, E. F. Susan, M. E. Anne and R. T. Rubin), pp. 1003-1027. San Diego: Academic Press.
Antonio, D. S. M., Guidugli-Lazzarini, K. R., do Nascimento, A. M., Simoes, Z. L. P. and Hartfelder, K. (2008). RNAi-mediated silencing of vitellogenin gene function turns honeybee (*Apis mellifera*) workers into extremely precocious foragers. *Naturwissenschaften* **95**, 953-961.
Azevedo, D. O., Zanuncio, J. C., Delabie, J. H. C. and Serrão, J. E. (2011). Temporal variation of vitellogenin synthesis in *Ectatomma tuberculatum* (Formicidae: Ectatomminae) workers. *J. Insect Physiol.* **57**, 972-977.
Barchuk, A. R., Bitondi, M. M. G. and Simões, Z. L. P. (2002). Effects of juvenile hormone and ecdysone on the timing of vitellogenin appearance in hemolymph of queen and worker pupae of *Apis mellifera*. *J. Insect Sci.* **2**, 1-8.
Bell, W. J. (1973). Factors controlling initiation of vitellogenesis in a primitively social bee, *Lasioglossum zephyrum* (Hymenoptera: Halictidae). *Insectes Soc.* **20**, 253-260.
Bloch, G., Borst, D. W., Huang, Z.-Y., Robinson, G., Cnaani, J. and Hefetz, A. (2000). Juvenile hormone titers, juvenile hormone biosynthesis, ovarian development and social environment in *Bombus terrestris*. *J. Insect Physiol.* **46**, 47-57.
Brent, C. and Vargo, E. (2003). Changes in juvenile hormone biosynthetic rate and whole body content in maturing virgin queens of *Solenopsis invicta*. *J. Insect Physiol.* **49**, 967-974.
Brent, C., Peeters, C., Dietmann, V., Crewe, R. and Vargo, E. (2006). Hormonal correlates of reproductive status in the queenless ponerine ant, *Streblognathus peetersi*. *J. Comp. Physiol. A* **192**, 315-320.
Burns, S., Teal, P. E. A., Vander Meer, R. K., Nation, J. and Vogt, J. (2002). Identification and action of juvenile hormone III from sexually mature alate females of the red imported fire ant, *Solenopsis invicta*. *J. Insect Physiol.* **48**, 357-365.
Camargo Mathias, M. I. and Caetano, F. H. (1995). Trophic eggs in workers of *Neoponera villosa* ants (Hymenoptera: Ponerinae). *J. Adv. Zool.* **16**, 62-65.
Champalbert, A. and Lachaud, J.-P. (1990). Existence of a sensitive period during the ontogenesis of social behaviour in a primitive ant. *Anim. Behav.* **39**, 850-859.
Chen, M.-E., Lewis, D. K., Keeley, L. L. and Pietrantonio, P. V. (2004). cDNA cloning and transcriptional regulation of the vitellogenin receptor from the imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). *Insect Mol. Biol.* **13**, 195-204.
Corona, M., Velarde, R., Remolina, S., Moran-Lauter, A., Wang, Y., Hughes, K. and Robinson, G. (2007). Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. *Proc. Natl. Acad. Sci. USA* **104**, 7128-7133.
Corona, M., Libbrecht, R., Wurm, Y., Riba-Grognuz, O., Studer, R. A. and Keller, L. (2013). Vitellogenin underwent subfunctionalization to acquire caste and behavioral specific expression in the harvester ant *Pogonomyrmex barbatus*. *PLoS Genet.* **9**, e1003730.
Cuvillier-Hot, V., Lenoir, A., Crewe, R., Malosse, C. and Peeters, C. (2004). Fertility signalling and reproductive skew in queenless ants. *Anim. Behav.* **68**, 1209-1219.
Dallacqua, R., Simões, Z. L. P. and Bitondi, M. M. G. (2007). Vitellogenin gene expression in stingless bee workers differing in egg-laying behavior. *Insectes Soc.* **54**, 70-76.
Davis, R. E., Kelly, T. J., Masler, E. P., Fescemyer, H. W., Thyagaraja, B. S. and Borkovec, A. B. (1990). Hormonal control of vitellogenesis in the gypsy moth, *Lymantria dispar* (L.): suppression of haemolymph vitellogenin by the juvenile hormone analogue, methoprene. *J. Insect Physiol.* **36**, 231-238.
Dhadialla, T. S., Hays, A. R. and Raikhel, A. S. (1992). Characterization of the solubilized mosquito vitellogenin receptor. *Insect Biochem. Mol. Biol.* **22**, 803-816.
Dietemann, V. and Peeters, C. (2000). Queen influence on the shift from trophic to reproductive eggs laid by workers of the ponerine ant *Pachycondyla apicalis*. *Insectes Soc.* **47**, 223-228.
Excels, W. (1974). Occurrence and significance of vitellogenins in female castes of social Hymenoptera. *Am. Zool.* **14**, 1229-1237.
Fénéron, R. and Billen, J. (1996). Ovarian cycle in *Ectatomma tuberculatum* workers (Formicidae, Ponerinae). *Invert. Reprod. Dev.* **29**, 79-85.
Fénéron, R., Durand, J. L. and Jaisson, P. (1996). Relation between behaviour and physiological maturation in a ponerine ant. *Behaviour* **133**, 791-806.
Fluri, P., Lüscher, M., Wille, H. and Gerig, L. (1982). Changes in weight of the pharyngeal gland and haemolymph titres of juvenile hormone, protein and vitellogenin in worker honey bees. *J. Insect Physiol.* **28**, 61-68.
Giorgi, F., Bradley, J. T. and Nordin, J. H. (1999). Differential vitellin polypeptide processing in insect embryos. *Micron* **30**, 579-596.

Giray, T., Giovanetti, M. and West-Eberhard, M. J. (2005). Juvenile hormone, reproduction, and worker behavior in the neotropical social wasp *Polistes canadensis*. *Proc. Natl. Acad. Sci. USA* **102**, 3330-3335.
Glinka, A. V. and Wyatt, G. R. (1996). Juvenile hormone activation of gene transcription in locust fat body. *Insect Biochem. Mol. Biol.* **26**, 13-18.
Gobin, B., Peeters, C. and Billen, J. (1998). Production of trophic eggs by virgin workers in the ponerine ant *Gnamptogenys menadensis*. *Physiol. Entomol.* **23**, 329-336.
Guidugli, K. R., Nascimento, A. M., Amdam, G. V., Barchuk, A. R., Omholt, S., Simões, Z. L. P. and Hartfelder, K. (2005). Vitellogenin regulates hormonal dynamics in the worker caste of a eusocial insect. *FEBS Lett.* **579**, 4961-4965.
Hagedorn, H. H. and Kunkel, J. G. (1979). Vitellogenin and vitellin in insects. *Annu. Rev. Entomol.* **24**, 475-505.
Hartfelder, K. (2000). Insect juvenile hormone: from "status quo" to high society. *Braz. J. Med. Biol. Res.* **33**, 157-177.
Hiremath, S. and Jones, D. (1992). Juvenile hormone regulation of vitellogenin in the gypsy moth, *Lymantria dispar*: suppression of vitellogenin mRNA in the fat body. *J. Insect Physiol.* **38**, 461-474.
Hora, R. R., Feneron, R., Valenzuela, J., Favila, M. E. and Fresneau, D. (2001). Queen size dimorphism in the ant *Ectatomma tuberculatum* (Hymenoptera: Formicidae: Ponerinae). *Sociobiology* **38**, 407-420.
Hora, R. R., Vilela, E., Fénéron, R., Pezon, A., Fresneau, D. and Delabie, J. (2005). Facultative polygyny in *Ectatomma tuberculatum* (Formicidae, Ectatomminae). *Insectes Soc.* **52**, 194-200.
Hora, R. R., Poteaux, C., Doums, C., Fresneau, D. and Fénéron, R. (2007). Egg cannibalism in a facultative polygynous ant: conflict for reproduction or strategy to survive? *Ethology* **113**, 909-916.
Hsieh, T. M. and Su, T. H. (2000). Effects of pyriproxyfen on ovaries of the pharaoh ant *Monomorium pharaonis* (Hymenoptera: Formicidae). *Plant Prot. Bull.* **42**, 73-82.
Huang, Z.-Y., Robinson, G. E., Tobe, S. S., Yagi, K. J., Strambi, C., Strambi, A. and Stay, B. (1991). Hormonal regulation of behavioural development in the honey bee is based on changes in the rate of juvenile hormone biosynthesis. *J. Insect Physiol.* **37**, 733-741.
Jassim, O., Huang, Z. Y. and Robinson, G. E. (2000). Juvenile hormone profiles of worker honey bees, *Apis mellifera*, during normal and accelerated behavioural development. *J. Insect Physiol.* **46**, 243-249.
Khila, A. and Abouheif, E. (2008). Reproductive constraint is a developmental mechanism that maintains social harmony in advanced ant societies. *Proc. Natl. Acad. Sci. USA* **105**, 17884-17889.
Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
Lewis, D. K., Campbell, J. Q., Sowa, S. M., Chen, M.-E., Vinson, S. B. and Keeley, L. L. (2001). Characterization of vitellogenin in the red imported fire ant, *Solenopsis invicta* (Hymenoptera: Apocrita: Formicidae). *J. Insect Physiol.* **47**, 543-551.
Libbrecht, R., Corona, M., Wende, F., Azevedo, D. O., Serrão, J. E. and Keller, L. (2013). Interplay between insulin signaling, juvenile hormone, and vitellogenin regulates maternal effects on polyphenism in ants. *Proc. Natl. Acad. Sci. USA* **110**, 11050-11055.
Michener, C. D. (1974). *The Social Behavior of the Bees: A Comparative Study*, 1st edn, 404 pp. Harvard University Press.
Morandin, C., Havukainen, H., Kulmuni, J., Dhaygude, K., Trontti, K. and Helantera, H. (2014). Not only for egg yolk: functional and evolutionary insights from expression, selection, and structural analyses of Formica ant vitellogenins. *Mol. Biol. Evol.* **31**, 2181-2193.
Nelson, C. M., Ihle, K. E., Fondrk, M. K., Page, R. E. and Amdam, G. V. (2007). The gene vitellogenin has multiple coordinating effects on social organization. *PLoS Biol.* **5**, e62.
Paes-de-Oliveira, V. T., Poiani, S. B., Antonialli, W. F., Jr and da Cruz-Landim, C. (2008). Morphometric changes on honeybee *Apis mellifera* L. workers fat body cells after juvenile hormone topic application at emergence. *Micron* **39**, 426-430.
Peeters, C. (1997). Morphologically 'primitive' ants: comparative review of social characters, and the importance of queen worker dimorphism. In *The Evolution of Social Behavior in Insects and Arachnids*. (ed. J. C. Choe and B. J. Crespi), pp. 372-391. Cambridge: Cambridge University Press.
Pfaffl, M. W., Horgan, G. W. and Dempfle, L. (2002). Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* **30**, e36.
Pinto, L. Z., Bitondi, M. M. G. and Simões, Z. L. P. (2000). Inhibition of vitellogenin synthesis in *Apis mellifera* workers by a juvenile hormone analogue, pyriproxyfen. *J. Insect Physiol.* **46**, 153-160.
Raikhel, A. S. and Dhadialla, T. S. (1992). Accumulation of yolk proteins in insect oocytes. *Annu. Rev. Entomol.* **37**, 217-251.
Robinson, G. E. and Vargo, E. L. (1997). Juvenile hormone in adult eusocial Hymenoptera: gonadotropin and behavioral pacemaker. *Arch. Insect Biochem. Physiol.* **35**, 559-583.
Ronnau, M., Azevedo, D. O., Fialho, M. d. C. Q., Gonçalves, W. G., Zanuncio, J. C. and Serrão, J. E. (2015). Changes in follicular cells architecture during vitellogenin transport in the ovary of social Hymenoptera. *Protoplasma* doi:10.1007/s00709-015-0843-0.
Röseler, P.-F., Röseler, I., Strambi, A. and Augier, R. (1984). Influence of insect hormones on the establishment of dominance hierarchies among foundresses of the paper wasp, *Polistes gallicus*. *Behav. Ecol. Sociobiol.* **15**, 133-142.
Rychlik, W. (2007). OLIGO 7 primer analysis software. *Methods Mol. Biol.* **402**, 35-59.
Seehuus, S.-C., Krekling, T. and Amdam, G. V. (2006). Cellular senescence in honey bee brain is largely independent of chronological age. *Exp. Gerontol.* **41**, 1117-1125.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76-85.
Sommer, K. and Hölldobler, B. (1995). Colony founding by queen association and determinants of reduction in queen number in the ant *Lasius niger*. *Anim. Behav.* **50**, 287-294.
Sommer, K., Hölldobler, B. and Rembold, H. (1993). Behavioral and physiological aspects of reproductive control in a *Diacamma* species from Malaysia (Formicidae, Ponerinae). *Ethology* **94**, 162-170.
Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876-4882.
Tian, H., Vinson, S. B. and Coates, C. J. (2004). Differential gene expression between alate and dealate queens in the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). *Insect Biochem. Mol. Biol.* **34**, 937-949.
Tufail, M. and Takeda, M. (2008). Molecular characteristics of insect vitellogenins. *J. Insect Physiol.* **54**, 1447-1458.
Tufail, M. and Takeda, M. (2009). Insect vitellogenin/lipophorin receptors: Molecular structures, role in oogenesis, and regulatory mechanisms. *J. Insect Physiol.* **55**, 88-104.
Wheeler, D., Liebig, J. and Hölldobler, B. (1999). Atypical vitellins in ponerine ants (Formicidae: Hymenoptera). *J. Insect Physiol.* **45**, 287-293.
Wurm, Y., Wang, J., Riba-Grognuz, O., Corona, M., Nygaard, S., Hunt, B. G., Ingram, K. K., Falquet, L., Nipitwattanaphon, M., Gotzek, D. et al. (2011). The genome of the fire ant *Solenopsis invicta*. *Proc. Natl. Acad. Sci. USA* **108**, 5679-5684.
Wyatt, G. R., Braun, R. P. and Zhang, J. (1996). Priming effect in gene activation by juvenile hormone in locust fat body. *Arch. Insect Biochem. Physiol.* **32**, 633-640.