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Research Article

Effect of juvenile hormone on phenoloxidase and hemocyte number: The role of age, sex, and immune challenge

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ABSTRACT

Hormones are key factors in determining the response of organisms to their environment. For example, the juvenile hormone (JH) coordinates the insects' development, reproduction, and survival. However, it is still unclear how the impact of juvenile hormone on insect immunity varies depending on the sex and reproductive state of the individual, as well as the type of the immune challenge (*i.e.*, Gram-positive or Gram-negative bacteria). We used *Tenebrio molitor* and methoprene, a JH analog (JHa) to explore these relationships. We tested the effect of methoprene on phenoloxidase activity (PO), an important component of humoral immunity in insects, and hemocyte number. Lyophilized Gram-positive *Staphylococcus aureus* or Gram-negative *Escherichia coli* were injected for the immune challenge. The results suggest that JH did not affect the proPO, PO activity, or hemocyte number of larvae. JH and immune challenge affected the immune response and consequently, affected adult developmental stage and sex. We propose that the influence of JH on the immune response depends on age, sex, the immune response parameter, and the immune challenge, which may explain the contrasting results about the role of JH in the insect immune response.

1. Introduction

Life history theory proposes that natural selection shapes the adaptation of organisms to the environment. When faced with a scarcity of resources, organisms prioritize one attribute over another, depending on their life stage (Caroci et al., 2004; Stearns, 1992). For example, whereas young organisms prioritize survival, mature adults improve reproduction at the cost of survival (Zera and Harshman, 2001). Hormones are key to understanding how the assignment of resources is controlled according to age and reproduction (Sirotkin, 2005). These factors are an essential part of the life history of vertebrates and invertebrates because they influence distinct aspects of physiology, morphology, growth, and behavior (Zera et al., 2007). For instance, in the bird *Taeniopygia guttata*, after hatching from eggs with high testosterone levels, the chicks grow faster. Upon reaching adulthood, the increase of testosterone in these organisms promotes greater reproductive success (Gil et al., 1999) and, at the same time, negatively affects the immune response of males but not females (Hau, 2007). In invertebrates, the juvenile hormone (JH) modifies key characteristics of their life histories: development, reproduction, and survival (Zhu et al., 2018; Flatt et al., 2005; Nijhout, 1998). Although this hormone increases fertility, it also reduces the immune response and enhance oxidative stress (Rantala et al., 2003; Contreras-Garduño et al., 2009; Martínez-Lendech et al., 2019). However, it is still unclear what effect the JH may have on the immune system in relation to sexual maturity, sexual dimorphism, or immune challenge (*i.e.*, challenge with Gram-positive or Gram-negative bacteria, fungus, or nylon implant). The JH has an important role in development and reproduction, but it is not known if JH affects the immune response

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differentially, according to developmental stage (larva *vs.* adult), sex (male *vs.* female), reproductive development (mature *vs.* immature adults), or the immune challenge (Gram-positive or Gram-negative bacteria).

Whether JH induces a positive or negative effect may depend on the developmental stage of the animals (Table 1). In larvae of *Bombyx mori* JH favors the production of the antimicrobial peptides (Tian et al., 2010). However, in larvae of *Spodoptera littoralis,* JH decreased the immune response against parasitoids (Khafagi and Hegazi, 2001). Finally, in larvae of *Tribolium castaneum*, JH did not affect the cellular immune response (Hepat and Kim, 2014). This positive, negative or null effect of the JH on the immune response reveal its complicated interaction and might be even more complicated if includes distinct pathogens (Table 1). For example, the application of JH is known to generate greater antimicrobial activity against Gram-negative (*Escherichia coli*) than Gram-positive (*Staphylococcus aureus*) bacteria in the larvae of *Bombyx mori* (Tian et al., 2010).

In adults positive and negative results are also found. Juvenile hormone downloads the expression of antimicrobial peptides in the post-eclosion phase of the female *Aedes aegypti* (Chang et al., 2021) but enhances phenoloxidase (PO) activity in females of the monarch butterfly *Danaus plexippus* (Villanueva et al., 2013). In *Drosophila melanogaster*, JH suppressed the antimicrobial peptide production in mated females and decreased its resistance against *Providencia rettgeri* compared with control females (Schwenke and Lazzaro, 2017). Additionally, the influence of JH on the immune response has been investigated in males (Rantala et al., 2003; Contreras-Garduño et al., 2009), females (Flatt et al., 2008; Pamminger et al., 2016), but has been rarely investigated in both sexes at the same time (Rantala et al., 2020; Table 1). JH's negative or positive effect on immune responses might be related to reproductive status or sexual dimorphism (Adamo et al., 2001). The JH has been postulated as a mediating mechanism between the signaling to produce secondary sexual characteristics of males (used to attract a mate) with a concomitant reduction in their immune response (Rantala et al., 2003; Contreras-Garduño et al., 2009). However, it has been proposed that different sex-specific hormones impact the sexual differences in immune response, and some controversy arises in insects (Kelly et al., 2018). So, it will be interesting to know if JH affects sexual dimorphism in insect immune responses. If JH is indeed a mediating mechanism, it would negatively affect the immune response of males but not that of females (Jacobs and Zuk, 2012).

Table 1 shows mixed results of the role of JH on immune response, and that there are no studies about the role of JH on immune response since larvae to adults. With these differences in methodology and species used, it is not possible to determine whether JH's positive or negative effect on the immune response is due to the reproductive status and/or sexual dimorphism (Table 1). Clarifying this question will be important to identify the mechanism of action of JH and the variation that might exist in the distinct life histories of each developmental stage, sex, and species (Jacobs and Zuk, 2012; Kim et al., 2020), as well as the immune challenge. However, no comprehensive study has considered these factors simultaneously in a single species (Table 1). Since the immune response, developmental stage, and reproduction in *Tenebrio molitor* are controlled by JH; we examined the positive, negative, or null impact of JH on the immune response (hemocyte number, prophenoloxidase and phenoloxidase activity) considering the insect development, sexual dimorphism, and the kind of immune challenge, specifically, Gram-positive or Gram-negative bacteria. In the following sections, we will describe the insects' immune response and the biology of *T. molitor*.

Insects combat pathogens with their humoral and/or cellular immune responses (Lemaitre and Hoffmann, 2007; Vigneron et al., 2019). The pro-phenoloxidase (proPO) activating system is part of the cellular and humoral response (Cammarata et al., 1996; Söderhäll and Cerenius, 1998; Vigneron et al., 2019). The cascade begins when the pathogens activate proteolytic enzymes, which activate the zymogen proPO (Johansson and Söderhäll, 1996). The latter is converted into phenoloxidase (PO), and this enzyme hydroxylates tyrosine to L-DOPA (L-3,4-dihydroxyphenylalanine). L-DOPA is oxidized to dopaquinone by PO or is decarboxylated to dopamine by L-DOPA decarboxylase (Huang et al., 2005; Park et al., 2010). The oxidation of dopaquinone by PO provides dopachrome, which is converted into 5,6- dihydroxyindole and then, the melanin is produced (Huang et al., 2005). By adhering to pathogens, melanin isolates them from the rest of the animal body, and they are then intoxicated by quinones (Söderhäll and Cerenius, 1998). In the cellular response, the adherence of hemocytes to a pathogen prepares it to be melanized and encapsulated (Vigneron et al., 2019; Krams et al., 2013, 2015). Even very large pathogens, such as parasitoids and nematodes, can be killed by encapsulation (Söderhäll and Cerenius, 1998; Vigneron et al., 2019).

In the mealworm beetle *T. molitor*, the larval stage lasts about 7–12 months, and the pupal stage around 10–15 days (unpublished data). After emerging as an adult, the insect matures sexually in 6–7 days (Tschinkel et al., 1967) and reaches its reproductive peak at day 12 after post-imaginal hatching (Cole et al., 2003). The most common form of JH depends on the insect species (Kamita and Hammock, 2010; Tsubota et al., 2010), and JH III is the only form present in *T. molitor* (Rantala et al., 2003). It promotes the production of male pheromones (Rantala et al., 2003), which attract females (Rantala et al., 2003; Pölkki et al., 2012), and JH favors pheromones at the expense of PO activity (Rantala et al., 2003). In adults, 10 μg of JH favors male survival against the fungus *Metarhizium robertsii* compared with females, but without JH addition, the females' survival was better than males' survival (Rantala et al., 2020). Regarding the immune response, in *T. molitor*, on the one hand, the IMD and Toll pathways lead to the production of antimicrobial peptides; on the other hand, the proPO cascade leads to melanin synthesis (Park et al., 2010). *T. molitor* hemocytes combat foreign enemies using nodulation, phagocytosis, and encapsulation (Vigneron et al., 2019). Their hemocytes engulf fungi or bacteria after opsonization with the Scavenger Receptor class C (Kim et al., 2017). Finally, it is well known that *T. molitor* is more susceptible to Gram-negative than Gram-positive bacteria (Dhinaut et al., 2018; Medina-Gómez et al., 2018). Hence, *T. molitor* is a good model species to investigate if the positive or negative effect of JH on immune response depends on the developmental stage, sex, and immune challenge, either Gram-negative (*Escherichia coli*) or Gram-positive (*Staphylococcus aureus*) bacterial elicitors.

Hormones coordinate multiple physiological processes linked with growth and maintenance that favors survival and reproduction. Consequently, they are the key factors involved in integrating different features of the life histories of animals (McGlothlin and Ketterson, 2008). In males of *Junco hyemalis*, testosterone is an example because it promotes the production of ornaments (white tail feathers) that are essential for female choice as well as male competition (McGlothlin et al., 2008) and aggression (Roberts et al., 2007), but reduce the immune response (Peterson et al., 2013). As far as we know, in animals, there are no reports of how hormones affect immune response simultaneously on the developmental stage, sexual dimorphism, and immune challenge (Table 1).

2. Materials and methods

2.1. Insects and experimental design

A total of 1937 *T. molitor* adults and larvae were used. The colony was maintained at 27 ± 0.5 °C in darkness (Márquez-García et al., 2016; Castro-Vargas et al., 2017) in the Evolutionary Ecology Lab at ENES-UNAM, Morelia Campus. Food was provided *ad libitum* and consisted of bran and cornmeal (3:1) with fresh apple slices added every other day (Márquez-García et al., 2016; Castro-Vargas et al., 2017). The food was sterilized (125 ± 2 °C for 15 min) to avoid infections (Castro-Vargas et al., 2017).

The experimental design consisted of larvae (2.0–2.5 cm on 11–12th

Table 1
Overview of reviewed sources on the effect of JH on immune response according to species, ages, sexes, and challenges. This table reveals a positive, negative or null effect of JH on the immune response. These contrasting results might be due to species, development, sex, immune challenge or the immune response parameter recorded,. However, any study has been carried out testing all of them simultaneously.

Species	Larva or pupa	Adult	Challenge	Effect of JH	Reference
Aedes aegypti	No	Females	*Beauveria bassiana* and *Enterobacter cloacae*	Negative (Diptericin, Cecropin A and Defensin A)	Chang et al. (2021)
Tenebrio molitor	No	Males and Females	*Metarhizium robertsii*	Negative on females but positive in males (Survival) Positive (Hemocyte, Defensin A and Cecropin A)	Rantala et al. (2020)
Aedes aegypti	Yes	No	*Escherichia coli*	Null (NO synthase, Gambicin and Attacin B)	Kim et al. (2020)
Drosophila melanogaster	No	Females	*Providencia rettgeri*	Negative (Cecropin A, Attacin A, Metchnikowin, Defensin and Diptericin A)	Schwenke and Lazzaro (2017)
Lasius niger	No	Females	*Metarhizium pingshaense*	Negative (proPhenoloxidase, Phenoloxidase) Positive (Defensin) Null (Cathepsinl)	Pamminger et al. (2016)
Glyphodes pyloalis	Larvae	Females	*Beauveria bassiana*	Negative (Nodulation)	Khosravi et al. (2014)
Tribolium castaneum	Yes	No	*Escherichia coli*	Negative (Hemocyte nodulation and hemocyte-spreading) Null (Nodules per larva) Positive in both sexes (Phenoloxidase)	Hepat and Kim (2014)
Danaus plexippus	No	Males and Females	No challenge	Positive in males (Hydrogen peroxide)	Villanueva et al. (2013)
Bombyx mori	Yes	No	*Escherichia coli* and *Staphilococcus aureus*	Null in both sexes (Lytic activity) Positive against *E. coli* (Gloverin-like protein 1 and 2, Morincin-1, Lebocin-3, Nuecin and Cecropin B)	Tian et al. (2010)
Calopteryx splendens	No	Males	*Serratia marcescens*	Negative (Phenoloxidase)	Contreras-Garduño et al. (2009)
Spodoptera exigua	Yes	No	No challenge	Negative (Plasmatocytes)	Kim et al. (2008)
Drosophila melanogaster	Yes	No	*Escherichia coli* and *Micrococcus luteus*	Negative (Drosomycin, Diptericin, Metchnikowin, Drosocin, Attacin-D and Defensin) Null (Cecropin-B, Drosomycin-5, Cecropin C, and A1, Andropin and Attacin-B)	Flatt et al. (2008)
Plutella xylostella	Yes	No	*Bacillus thuringiensis*	Negative (Hemocyte-spreading behavior)	Kwon and Kim (2007)
Rhodnius prolixus	No	Males	*Escherichia coli*	Negative (Phenoloxidase)	Nakamura et al. (2007)
Neobellieria bullata	Yes	No	Laminarin	Negative (Nodulation)	Franssens et al. (2006)
Pseudoplusia includens	Yes	No	No challenge	Negative (Plasmatocytes)	Clark et al. (2005)
Apis mellifera	No	Adults (Non-reproductive)	No challenge	Null (Hemocyte number)	Amdam et al. (2004)
Tenebrio molitor	No	Males	Nylon implant	Negative (Phenoloxidase and Melanization) Null (Lytic activity)	Rantala et al. (2003)
Tenebrio molitor	No	Adults	No challenge	Negative (Phenoloxidase)	Rolff and Siva-Jothy (2002)
Spodoptera littoralis	Yes	No	*Microplitis rufiventris*	Negative as increased the dosis of JH (Melanization)	Khafagi and Hegazi (2001)
Apis mellifera	Yes	No	No challenge	Positive (Phenoloxidase)	Bitondi et al. (1998)
Manduca sexta	Yes	No	No challenge	Negative (Phenoloxidase and Melanization)	Hiruma and Riddiford (1988)

instar) and adults (2 or 12-day-old females and males, were sexed in the pupal stage according to Bhattacharya et al. (1970) and treated with: 1) methoprene, a juvenile hormone analogue, diluted in acetone (JHa group) that is the solvent in which methoprene is most stable (Flatt and Kawecki, 2007), or only 2) acetone (control group). Five mg of methoprene (Sigma, Aldrich, St. Louis, M) were dissolved in 1 mL of acetone (99.5%; Sigma-Aldrich, St. Louis, MO), and 1 μL of that solution was added to 999 μL of acetone, resulting in a final JHa concentration of 5 ng/μL (Martínez-Lendech et al., 2019; Contreras-Garduño et al., 2009). Using a micropipette of ten microliters, we applied 2 μL of the methoprene-acetone mixture (10 ng of JHa/per organism) on the top of the thorax near the head (close to the *corpora allata*, the insect organ where JH is released) (Contreras-Garduño et al., 2009). We did not use a naïve group because naïve and control groups treated with acetone did not differ in proPO or PO activity (Contreras-Garduño et al., 2009, 2011).

The larvae and adults were challenged by injection within one of three groups: 1) Gram-positive bacteria *Staphylococcus aureus*, 2) Gram-negative bacteria *Escherichia coli*, and 3) Phosphate Buffered Saline (PBS 1×, pH 7.4; Sigma) for the control group. In this way, six groups were established for each age and sex (see Fig. 1). In all injections, we used sterilized Hamilton syringes (10 μL). JHa, acetone, and the immune challenge were applied simultaneously, and after 3 h, we obtained the hemolymph (see below).

2.2. Immune challenge

We carried out pilot experiments to establish the doses of *E. coli* (pHrodo, Invitrogen; cat. P35360) or *S. aureus* (pHrodo, Invitrogen; cat. P35382) against *T. molitor*. We used 1 mL of sterile PBS in each glass container with 2 mg of bacteria. The control group was injected with PBS. Beetles of each sex were injected through the intersegmental membrane of the third pair of legs coxae. After 3 h of injecting at the same time the immune challenge plus JHa or acetone, we obtained a drop of hemolymph to analyze the protein content and immune response. We knew that this time is a good estimation to measure the PO activity because Contreras-Garduño et al. (2009) and Rantala et al. (2003) showed that JH reduces PO at 3 h, while Villanueva et al. (2013) showed that the effect of JH on PO activity lasts for >6 h.

2.3. Hemolymph extraction and hemocytes number

Hemolymph was extracted 3 h after the injection of bacteria and the addition of JHa. Insects were chilled on ice, and then we made an

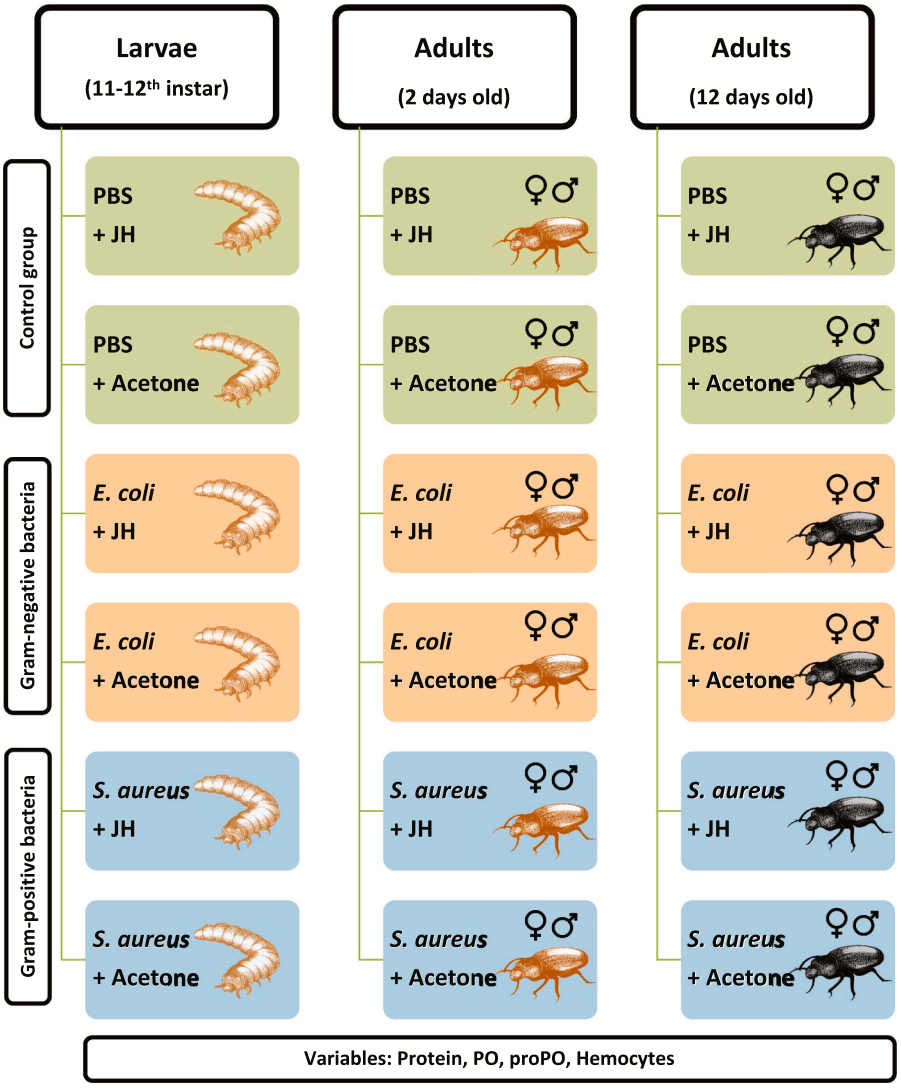

Fig. 1. Experimental design to test the effect of JHa on the proPO and PO activity and hemocytes number according to age (larvae or adults of 2 or 12 days old), immune challenge (*E. coli* or *S. aureus*), and sex (males or females of 2 or 12 days old). As control groups, we used insects injected with acetone because it was the solvent of JHa. The sample size is shown in Table 2.

incision between the head and the thorax to obtain 5 μL of hemolymph. This hemolymph was obtained with a micropipette (10 μL) and deposited in 1.5 mL (Axigen) vials previously pre-cooled with 300 μL of PBS. Samples were stored at −70 °C until the protein, proPO, and PO analyses.

We carried out another experiment similar to the above but, in this case, instead of taking into account hemolymph to record proteins, proPO, and PO, 348 insects, were used to obtain hemolymph to record the hemocyte number. In short, 5 μL of hemolymph was mixed with 5 μL of PBS. The mixture was added to a TC20 slide counter, and the slide was inserted into a TC20 automated cell counter (BioRad). After 30 s per reading, the apparatus recorded the cell number per sample.

2.4. Protein content, proPO, and PO activity

The commercial BCA protein assay kit (Thermo Scientific) was used to quantify the amount of protein per sample, following the manufacturer's instructions. In short, we used nine out of a 96 plate wells for the standard curve of protein with BSA (Bovin Serum Albumin). Each of these wells received 40 μL of Phosphate Buffered Saline, pH 7.4 (PBS; Sigma), 150 μL of the working reagent (WR), and 40 μL of BSA that ranged from 0 to 8000 μg/mL of protein. The other 87 wells were filled with 40 μL of PBS plus 150 μL of the WR reagent and 10 μL of the sample. The absorbance was read at 562 nm (Varioskan Flash Multimode Reader, Thermo Scientific) after 15 min of incubation at room temperature. We obtained the protein concentration in μg/mL of each sample with the equation of the linear regression curve made with the standard range. The PO activity was measured spectrophotometrically by recording dopachrome formation from *L*-dihydroxyphenylalanine (L-DOPA, Sigma) (Ardia et al., 2012; Contreras-Garduño et al., 2007). A volume per sample containing 40 μg/μL of protein was dose-titer until reaching 150 μL with PBS. These 150 μL were mixed with 50 μL of L-DOPA (4 mg/mL), obtaining a final volume of 200 μL. Samples were incubated for 30 min at room temperature inside each 96-well plate (Corning) well and subsequently read in a microplate reader at 490 nm every 5 min for one hour in an ELISA reader (Varioskan Flash Multimode Reader, Thermo Scientific). We verified that during this time, from 5 to 60 min, the proPO and PO activity was linear. As blanks, in 3 wells, we mixed 150 μL of PBS and 50 μL of L-DOPA. Placing PBS in the control wells allowed us to differentiate the absorbance reading in wells with and without samples. In addition, each 'treatment*pathogen*age*sex' group had a replica of 9 individuals. The enzyme activity was expressed as the rate of change of the optical density in time (Ardia et al., 2012). To measure the total PO activity (proPO), we added 5 μL of chymotrypsin (5 mg/mL) to the sample (with 40 μg/μL of protein) and then diluted it with PBS to 150 μl. Both proPO and PO were linear over time, and rates were measured over one hour and expressed as Activity. All treatments were split and included in a single plate to improve reproducibility. Our method was modified from Ardia et al. (2012). We did not use the inhibitor of melanization, we activated the proPO with chymotrypsin and PO with L-DOPA. In addition, we used PBS instead of cacodylate buffer.

2.5. Statistical analysis

The Shapiro-Wilk Normality Test was performed for each variable, but none had a normal distribution. The Hemocyte number variable was logarithm transformed and fitted to a normal distribution ($W = 0.99$, $p = 0.06$), while the variables PO and proPO were added 1 to fit them to a Gamma distribution. For the proPO and PO variables, an additional replica was performed and are reported as activity. Generalized Linear Models (Gaussian-link = "identity" and Gamma-link = "inverse") was performed to determine the differences between groups and interactions, followed by a Bonferroni test for multiple comparisons of means. The factors "Treatment", "Age", "Immune challenge", and "Sex" were included for each variable to determine the effect of JHa on the development, immune challenge, or sexual dimorphism on the immune response. All analyzes were performed in the RStudio V2022.07.1.554 program. Table 2 shows all means ± standard errors (mean ± SE).

3. Results

3.1. proPhenoloxidase

We found a similar proPO activity between control and JHa treatments (GLM Gamma-link = "inverse", $F = 0.16$, $p = 0.69$; Fig. 2; Table 3). However, the proPO activity was different according to immune challenge (GLM Gamma-link = "inverse", $F = 15.05$, $p < 0.0001$; Fig. 2; Table 3): PBS had more proPO activity than *E. coli* ($p < 0.0001$) and *S. aureus* ($p = 0.007$) and *S. aureus* showed more proPO activity than *E. coli* ($p = 0.05$). We also found differences according to age (GLM Gamma-link = "inverse", $F = 14.83$, $p < 0.0001$; Fig. 2; Table 3): larvae (11–12th instar) had more proPO activity than sexually immature (2 days old: $p < 0.0004$) or mature (12 days old: $p < 0.0001$) adults. Finally, we found differences according to sex (GLM Gamma-link = "inverse", $F = 3.98$, $p = 0.04$; Fig. 2; Table 3): females had more proPO activity than males ($p = 0.04$). The JHa did not affect proPO activity according to age ($F = 0.05$, $p = 0.95$; Fig. 2; Table 3) sex ($F = 0.15$, $p = 0.69$; Fig. 2; Table 3), and immune challenge ($F = 1.82$, $p = 0.16$; Fig. 2; Table 3).

In addition, at 2 days old, females challenged with *S. aureus* had more proPO activity than males in those sexes treated ($p < 0.0001$) or not ($p = 0.002$) with JHa (Fig. 2; Table 4). At 12 days old, control males and females had similar proPO activity ($p = 0.22$), but at the same age, JHa favored sexual dimorphism because males showed more proPO activity than females ($p = 0.005$; Fig. 2; Table 4).

3.2. Phenoloxidase

The PO activity was similar according to the treatments control and JHa (GLM Gamma-link = "inverse", $F = 0.0001$, $p = 0.99$; Fig. 3; Table 3) and sex (GLM Gamma-link = "inverse", $F = 0.53$, $p = 0.47$; Fig. 3; Table 3). However, we found differences according to immune challenge (GLM Gamma-link = "inverse", $F = 37.43$, $p < 0.0001$; Fig. 3; Table 3): PBS ($p < 0.0001$) and *S. aureus* ($p < 0.0001$) had more PO activity that *E. coli*, but PBS was similar than *S. aureus* ($p = 0.11$). Age was also different (GLM Gamma-link = "inverse", $F = 4.49$, $p = 0.01$; Fig. 3; Table 3): larvae (11–12th instar) had more PO activity than sexually immature (2 days old: $p = 0.02$) or mature (12 days old: $p = 0.01$) adults. In PO activity we found an effect of JHa on the immune challenge (GLM, Gamma-link = "inverse", $F = 3.94$, $p = 0.01$): JHa + *S. aureus* had more PO activity than JHa + *E. coli* ($p = 0.02$). The JHa did not affect PO activity according to age ($F = 0.64$, $p = 0.53$; Fig. 3; Table 3) or sex ($F = 0.54$, $p = 0.46$; Fig. 3; Table 3).

As in proPO, we found and effect of JHa on sexual dimorphism in PO, but regarding immune challenge. In 2 days old adults, with *E. coli*, we did not find sexual differences with ($p = 0.61$; Fig. 3; Table 4) or without ($p = 0.98$; Fig. 3; Table 4) JHa. However, in sexually reproductive adults at 12 days old, males had more PO activity than females if both were challenged with *E. coli* and were treated with JHa ($p = 0.03$; Fig. 3; Table 4). No sexual differences were found in control males and females at 12 days old challenged with *E. coli* ($p = 0.26$; Fig. 3). Regarding immune challenge with *S. aureus*, no sexual differences were found in 12 days adults treated ($p = 0.48$; Fig. 3; Table 4) or not ($p = 0.19$; Fig. 3; Table 4) with JHa. However, JHa again affected sexual differences but at 2 days old: females treated with JHa had more PO activity than males ($p = 0.0001$; Fig. 3; Table 4), but no differences between sexes were found in control insects treated with *S. aureus* ($p = 0.07$; Fig. 3; Table 4).

3.3. Hemocytes number

Hemocytes number was not different between the treatments control and JHa (GLM gaussian-link = "identity", $F = 0.53$, $p = 0.47$; Fig. 4;

Table 2
Mean (± SE) and sample size for proPO, PO, and hemocytes number according to treatment.

Treatment	Challenge	Sex/Age	proPO mean (± SE), n	PO mean (± SE), n	Hemocytes number cells/uL mean (± SE), n
Control	**PBS**	Larvae	0.0500 (0.0045), 39	0.0419 (0.0039), 40	4535.7 (904.0), 19
		Females 2 days	0.0414 (0.0030), 43	0.0331 (0.0029), 43	1293.6 (371.2), 10
		Females 12 days	0.0369 (0.0031), 62	0.0350 (0.0026), 79	1270.8 (496.3), 4
		Males 2 days	0.0429 (0.0030), 55	0.0343 (0.0027), 58	1365.0 (536.5), 10
		Males 12 days	0.0354 (0.0043), 47	0.0392 (0.0040), 63	3202.7 (974.3), 12
	E. coli	Larvae	0.0248 (0.0049), 17	0.0120 (0.0047), 17	3130.3 (820.4), 19
		Females 2 days	0.0259 (0.0038), 25	0.0161 (0.0038), 25	789.4 (118.0), 14
		Females 12 days	0.0207 (0.0049), 20	0.0143 (0.0028), 37	2889.7 (702.5), 9
		Males 2 days	0.0253 (0.0040), 21	0.0159 (0.0039), 21	872.4 (245.3), 11
		Males 12 days	0.0301 (0.0056), 20	0.0203 (0.0053), 37	2357.1 (1133.0), 9
	S. aureus	Larvae	0.0514 (0.0058), 20	0.0501 (0.0054), 20	1471.0 (226.9), 20
		Females 2 days	0.0437 (0.0039), 28	0.0344 (0.0042), 28	1155.1 (407.9), 12
		Females 12 days	0.0326 (0.0039), 41	0.0299 (0.0037), 41	2167.5 (423.7), 10
		Males 2 days	0.0238 (0.0039), 29	0.0227 (0.0037), 29	1540.7 (372.4), 12
		Males 12 days	0.0234 (0.0041), 33	0.0229 (0.0045), 33	2166.0 (951.9), 14
JHa	**PBS**	Larvae	0.0502 (0.0053), 40	0.0420 (0.0042), 40	4634.0 (1802.9), 12
		Females 2 days	0.0393 (0.0037), 49	0.0341 (0.0033), 49	1118.6 (333.8), 11
		Females 12 days	0.0353 (0.0036), 59	0.0312 (0.0026), 76	2546.3 (799.5), 9
		Males 2 days	0.0348 (0.0029), 52	0.0303 (0.0025), 54	1200.8 (333.2), 11
		Males 12 days	0.0304 (0.0037), 49	0.0298 (0.0030), 66	3011.6 (656.6), 8
	E. coli	Larvae	0.0322 (0.0053), 19	0.0088 (0.0035), 19	1714.8 (326.2), 12
		Females 2 days	0.0282 (0.0046), 20	0.0191 (0.0046), 20	1095.1 (282.4), 11
		Females 12 days	0.0160 (0.0039), 17	0.0221 (0.0034), 34	2660.7 (1042.2), 10
		Males 2 days	0.0339 (0.0062), 20	0.0228 (0.0046), 20	1464.8 (457.2), 11
		Males 12 days	0.0384 (0.0083), 20	0.0325 (0.0054), 37	3406.0 (999.9), 9
	S. aureus	Larvae	0.0451 (0.0041), 20	0.0410 (0.0050), 20	2266.3 (372.7), 15
		Females 2 days	0.0474 (0.0049), 26	0.0446 (0.0042), 26	1036.5 (230.2), 11
		Females 12 days	0.0324 (0.0042), 42	0.0300 (0.0042), 43	2097.8 (446.1), 9
		Males 2 days	0.0202 (0.0039), 30	0.0215 (0.0037), 30	699.7 (103.2), 12
		Males 12 days	0.0276 (0.0048), 31	0.0262 (0.0044), 31	610.9 (113.0), 11

Table 3) or according to sex (GLM gaussian-link = "identity", F = 0.33, p = 0.56; Fig. 4; Table 3). However, we found differences according to immune challenge (GLM gaussian-link = "identity", F = 3.91, p = 0.02; Fig. 4; Table 3): PBS had more hemocytes number than *S. aureus* (p = 0.002). In addition, we found differences according to age (GLM gaussian-link = "identity", F = 17.44, p < 0.0001; Fig. 4; Table 3): larvae (11–12th instar) had more hemocytes number than adults at 2 days old (p < 0.0001) but were similar than adults at 12 days old (p = 0.32), there also, 12 days old adults had more hemocytes number than adults at 2 days old (p = 0.0001). The JHa did not affect hemocytes number according to age (F = 0.02, p = 0.98; Fig. 4; Table 3) or sex (F = 0.04, p = 0.83; Fig. 2; Table 3) and immune challenge (F = 0.42, p = 0.66; Fig. 4; Table 3).

Finally, we found an effect of JHa on sexual differences according to immune challenge. No sexual differences were found between sexes treated (p = 0.29; Fig. 4; Table 4) or not (p = 0.42; Fig. 4; Table 4) with JHa and challenged with *S. aureus* at 2 days old. However, at 12 days old, females treated with JHa had more hemocyte number than males (p = 0.02; Fig. 4; Table 4) and no sexual differences were found at the same age but in the control group (p = 0.99; Fig. 4; Table 4). In the control group, at 12 days old, males had more hemocytes than females (p = 0.99; Fig. 4; Table 4), but after treated with JHa, only in females increased the hemocyte number and hence, no differences were found with males (p = 0.04; Fig. 4; Table 4).

4. Discussion

Results show that JHa did not affect the proPO and PO activity, or hemocyte number of larvae. JHa and immune challenge affected the immune response according to affected developmental stage and sex. We discuss our results considering the effect of JH on proPO, PO, and hemocyte number.

4.1. Age, sexual dimorphism, and immune challenge without the effect of JHa

In insects, the proPO/PO pathway (Adamo et al., 2001; Thomson and Sin, 1970; Trauer and Hilker, 2013) and hemocyte number (King and Hillyer, 2013; League et al., 2017) decrease with age. Our results are also consistent with other studies in which antimicrobial activity,

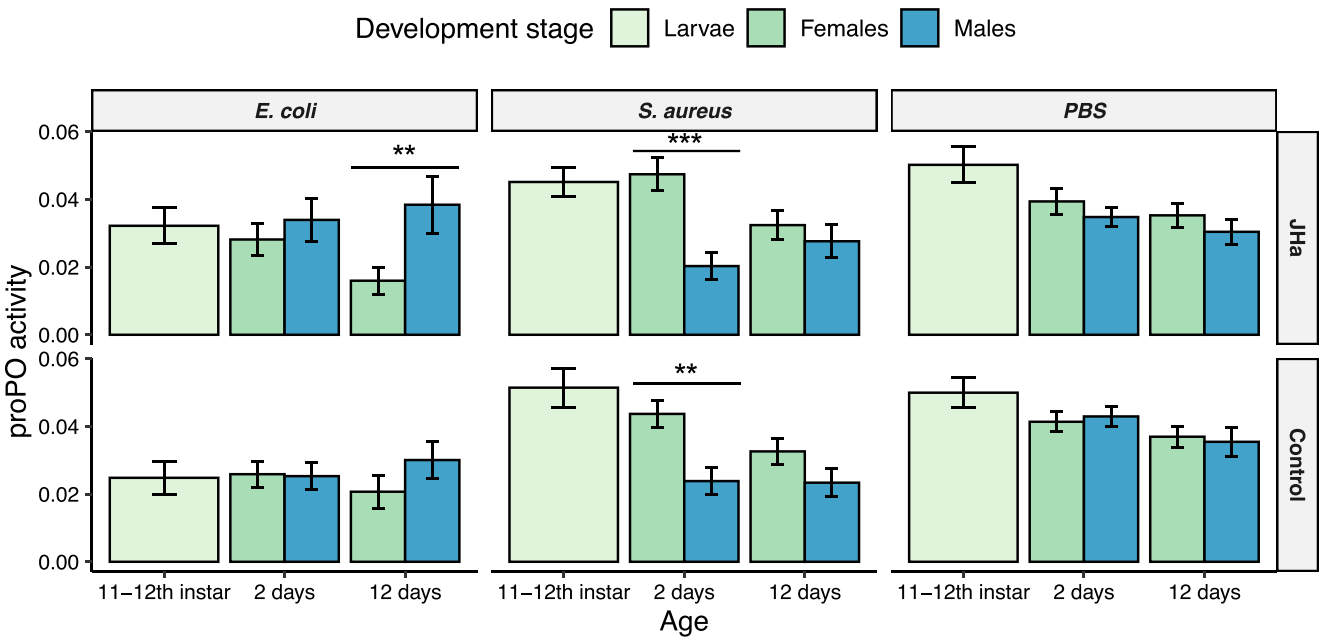

Fig. 2. ProPO activity. Here we show the effect of JH on prophenoloxidase activity as a function of immune challenge, sex, and age. The mean ± SE is shown in Table 2, and the pairwise comparisons are shown in Table 4.

Table 3
The differences across groups are sumarized. The immune parameter was higher >, lower <, or similar =, according to the treatment, challenge, age, and sex.

Immune parameters	Treatment (Control, JHa)	Challenge (*E. coli*, *S. aureus*, PBS)	Age (11–12th instar, 2 days, 12 days)	Sex (Females, Males)
Hemocytes	Control = JHa	PBS = *E. coli* PBS > *S. aureus* *S. aureus* = *E. coli*	2 days <11–12th instar 12 days = 11–12th instar 12 days >2 days	Females = Males
proPO	Control = JHa	PBS > *E. coli* PBS > *S. aureus* *S. aureus* > *E. coli*	2 days <11–12th instar 12 days <11–12th instar 12 days = 2 days	Females > Males
PO	Control = JHa	PBS > *E. coli* PBS = *S. aureus* *S. aureus* > *E. coli*	2 days <11–12th instar 12 days <11–12th instar 12 days = 2 days	Females = Males

hemocyte number, and PO activity decrease with age (Beetz et al., 2008; Booth et al., 2015). Life-history theory suggests a priority in the investment of resources into the immune function during immature stages to improve survival and a later decline in adults caused by the assignment of resources to favor reproduction at the expense of the immune response (Rantala et al., 2003). Since reproduction is essential in sexually mature stages, we expected investment in reproduction to be prioritized over the immune response (Lawniczak et al., 2007; Schwenke et al., 2016). In support of this hypothesis, the proPO and PO activity had higher activity in the larvae of *T. molitor* than in adults. However, there were no significant differences between larvae and 12 days adults in hemocyte number. This suggests that not all immune response decline according to age, but that might be reconfigured. In this case, adults of 12 days old might opt for hemocyte number rather than PO. The hypothesis of immune reconfiguration states that under stressful conditions, hormones reconfigure the immune response to eliminate the enemies, which may lead the host to get the maxim benefits with fewer costs (Adamo, 2014). This might explain our contrasting results between proPO, PO and hemocytes. Further work considering other immune response parameters such as antimicrobial peptides, nitric oxide, and reactive oxygen species are needed, to know which immune components change according to JH, if such effect is broad on the immune response, and finally, the role of other hormones such as octopamine, which is implicated in stress and the immune reconfiguration (Adamo, 2014) should be considered. Although hemocyte number has been recorded to test the effect of JH on immune response (*i.e.* Kim et al., 2020; Chang et al., 2021), it is important to consider that total circulating hemocyte number might be difficult to interpret as measurement of immune response if they are not related with parasite elimination. Hence, specific hemocyte type numbers (i.e. granulocyes) and activity (i.e. phagocytosis) should be also recorded.

Life-history theory predicts that in sexually mature adults, females should assign more resources to the immune response than males (Adamo et al., 2001; Rolff et al., 2005; Laughton et al., 2011). Accordingly, males invest more resources in the elaboration of secondary sexual characteristics (*e.g.*, horns, jawbones, singing, dancing, and pheromones) at the expense of the immune response (Zuk and Stoehr, 2002), while females must invest in the immune response if it is positively correlated with the production of eggs (Rolff and Siva-Jothy, 2002). However, our results do not support the previous hypotheses because no differences were found between sexes in PO nor hemocyte number (bet see below taking into account the immune challenge). We did not find significant differences between sexes before males invested in pheromone production (2-days old) compared with sexually mature males of 12-days old. However, the sexual dimorphism should not be

Table 4
Effect of the interaction between JHa and pathogen species on sex and developmental stage (pairwise comparisons by Treatment*Challenge*Age*Sex*).

Immune parameters	Treatment	Challenge	Age	Sex	Bonferroni test *p*-value
proPO	Control	*E. coli*	12-days-old	Females *vs.* Males	0.22
	JHa	*E. coli*	12-days-old	Females *vs.* Males	0.005*
	Control	*S. aureus*	2-days-old	Females *vs.* Males	0.002*
	JHa	*S. aureus*	2-days-old	Females *vs.* Males	< 0.0001*
PO	Control	*E. coli*	12-days-old	Females *vs.* Males	0.26
	JHa	*E. coli*	12-days-old	Females *vs.* Males	0.03*
	Control	*E. coli*	2-days-old	Females *vs.* Males	0.98
	JHa	*E. coli*	2-days-old	Females *vs.* Males	0.61
	Control	*S. aureus*	12-days-old	Females *vs.* Males	0.19
	JHa	*S. aureus*	12-days-old	Females *vs.* Males	0.48
	Control	*S. aureus*	2-days-old	Females *vs.* Males	0.0001*
	JHa	*S. aureus*	2-days-old	Females *vs.* Males	0.07
Hemocytes	Control	*S. aureus*	12-days-old	Females *vs.* Males	0.99
	JHa	*S. aureus*	12-days-old	Females *vs.* Males	0.02*
	Control	*S. aureus*	2-days-old	Females *vs.* Males	0.42
	JHa	*S. aureus*	2-days-old	Females *vs.* Males	0.29
	Control	PBS	12-days-old	Females *vs.* Males	0.04*
	JHa	PBS	12-days-old	Females *vs.* Males	0.69
	Control	PBS	2-days-old	Females *vs.* Males	0.89
	JHa	PBS	2-days-old	Females *vs.* Males	0.84

discarded before confronting males and females to at least one reproductive event and comparing its immune response (PO and hemocyte number) with the immune response of 2-day male and female adults. This is because, without a costly task, the differences in immune response might be mascaraed (Valtonen and Rantala, 2012). In addition, females showed more proPO than males, suggesting that under stressful conditions, they might respond better than males, producing more PO. However, this is an open question.

Immune response in invertebrates was previously considered to be non-specific and relatively simple, but recent studies have shown a complex immune response (Siva-Jothy et al., 2005; Rowley and Powell, 2007; Lanz-Mendoza and Contreras-Garduño, 2022) influenced by a pathogen (Schmid-Hempel, 2011) and JH (Tian et al., 2010; Rantala et al., 2020). The measurement of immune defenses can be complicated because different pathogens activate distinct immune cascades; such cascades are interconnected (Seppälä and Leicht, 2013; Contreras-Garduño and Lazcano-Canales, 2014). In addition, the interactions between two or more physiological systems sometimes result in changes in the transmission of the disease, and this neuro-immunoendocrine network adds yet greater complexity to the immune response (Demas et al., 2012). The immune challenges (*E. coli* and *S. aureus*) revealed fewer proPO than the control group (PBS), less PO activity in *E. coli* than PBS, and less hemocyte number in *S. aureus* than PBS. Although this is contradictory, it is possible that the immune response was a shift to antimicrobial activity, such as lysozyme activity and antimicrobial peptides. It is known that proPO pathway and lysozyme activity are traded-off (Rantala et al., 2003; Rao et al., 2010). This may explain our finding because a similar result has been observed in *T. molitor* (Ardia et al., 2012). A future study should test the activation of, for example, antimicrobial peptides, lysozyme activity, reactive oxygen, and nitrogen production.

Interestingly, proPO and PO activities were more elevated against *S. aureus* than *E. coli*. This is consistent with previous findings that propose that *T. molitor* is more susceptible to Gram-negative than Gram-positive bacteria (Dhinaut et al., 2018; Medina-Gómez et al., 2018). Given that hemocyte number was not different between immune challenges with *S. aureus* and *E. coli*, we propose that the proPO pathway rather than hemocyte number might be related to the resistance of *T. molitor* to Gram-positive bacteria.

4.2. Age, sexual dimorphism, and immune challenge with the effect of JHa

On the one hand, at 2 days old, challenged males with *S. aureus* had more proPO but less PO than females (males had less activated enzyme to produce melanin). However, no differences were found in the hemocyte number at the same age. Against *E. coli*, no differences were found in proPO, PO, and hemocyte number. Hence, the immune response may be affected by the interaction between JHa and the bacteria that exert more selective pressure on *T. molitor*: the Gram-positive bacteria rather than the Gram-negative bacteria. On the other hand, at 12 days old, males treated with JHa and challenged with *S. aureus* had more proPO than females, but no differences were found in PO, and females had more hemocytes than males. In addition, against *E. coli*, males had more PO than females, but JHa favored the females' hemocyte number. A previous study showed a differential effect of JH on antimicrobial activity according to challenge with *E. coli* or *S. aureus* (Tian et al., 2010). However, our results also suggest a shift of immune response parameters according to immune challenges due to the effect of JHa in sexually mature insects. Compared with larvae, the investment in reproduction in *T. molitor* adults, would likely occur at the expense of investment in the defense against pathogens, with JH as a potential modulating mechanism, depending on the immune response parameter. The exact molecular mechanism should be tested further.

Previous reports have found a mixed effect of JH on the immune response, being negative or positive, but in these studies, different life stages have been used (Rantala et al., 2003; Contreras-Garduño et al., 2009; Tian et al., 2010; Villanueva et al., 2013; Table 1). Here we hypothesized that the life stage might explain the such apparent mixed effect, the JH effect null or positive in larvae and negative in sexually mature adults. Life-history theory predicts that in sexually mature adults, females should assign more resources to the immune response than males (Adamo et al., 2001; Rolff et al., 2005; Laughton et al., 2011). Accordingly, males invest more resources in the elaboration of secondary sexual characteristics (*e.g.*, horns, jawbones, singing, dancing,

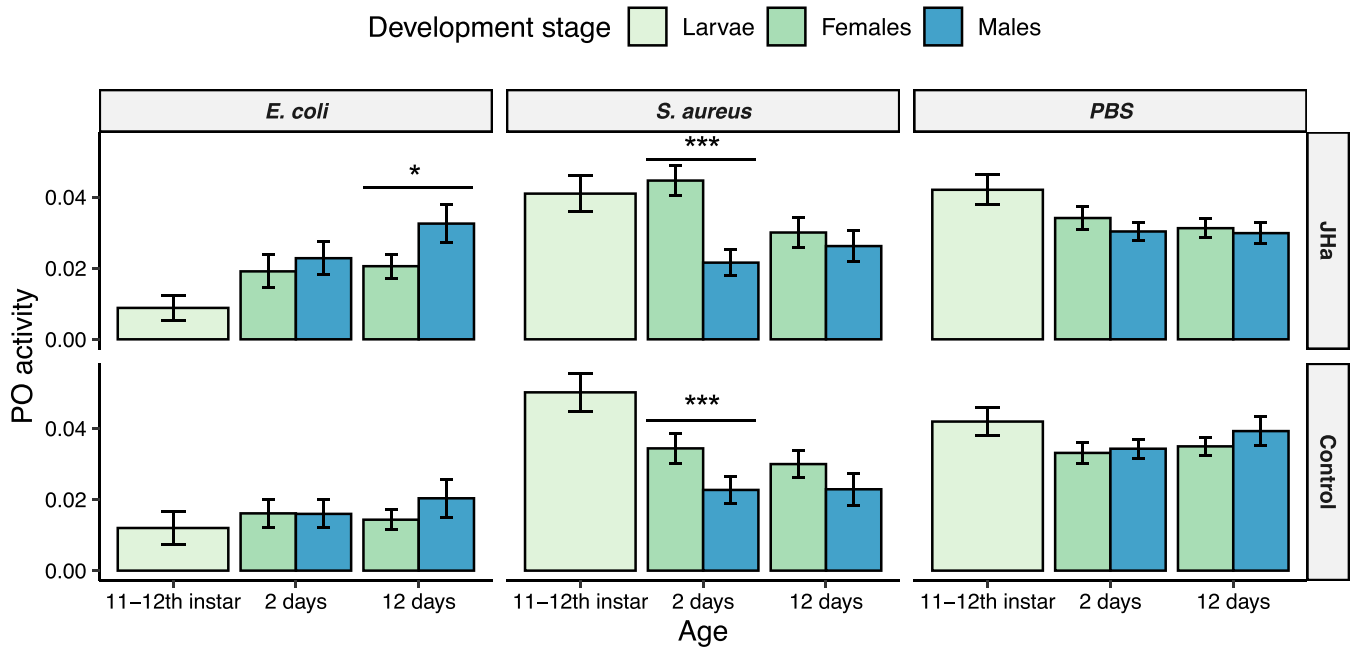

Fig. 3. PO activity. Here we show the effect of JH on phenoloxidase activity as a function of immune challenge, sex, and age. The mean ± SE is shown in Table 2, and the pairwise comparisons are shown in Table 4.

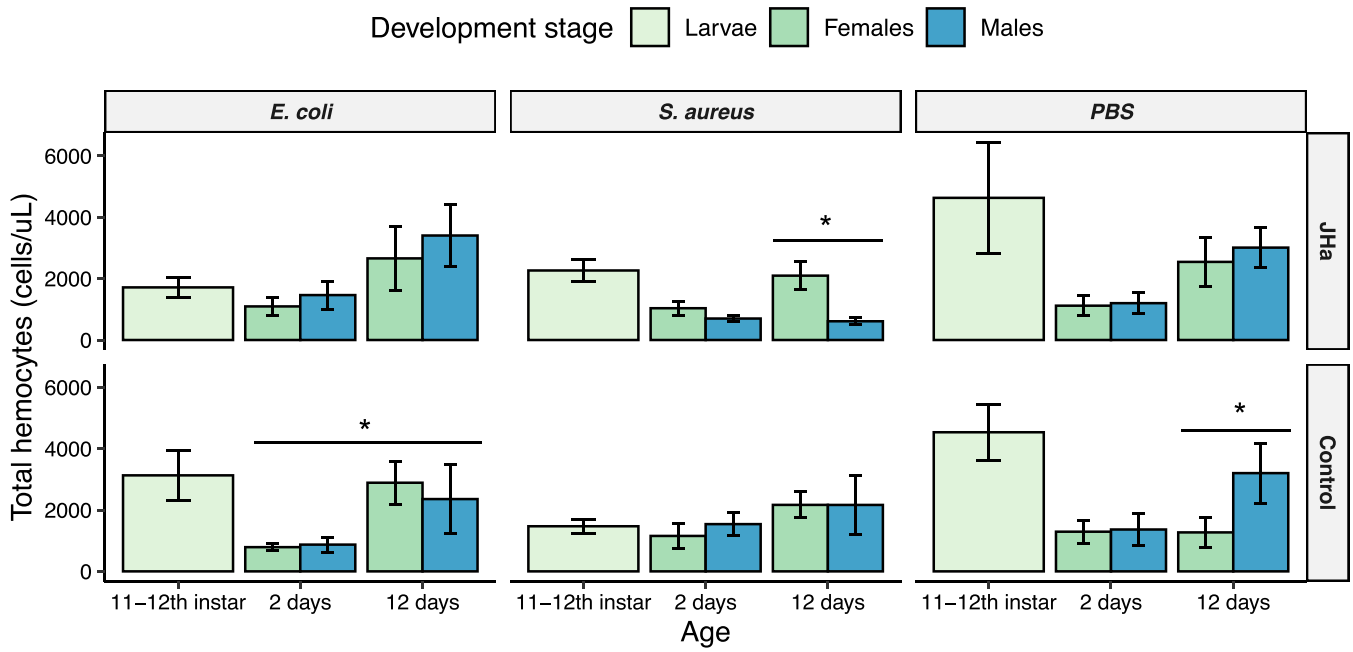

Fig. 4. Hemocytes number. Here we show the effect of JH on the total hemocyte number as a function of immune challenge, sex, and age. The mean ± SE is shown in Table 2, and the pairwise comparisons are shown in Table 4.

and pheromones) at the expense of the immune response (Zuk and Stoehr, 2002), while females must invest in the immune response if it is positively correlated with the production of eggs (Rolff and Siva-Jothy, 2002). The PO activity is lower in sexually mature *versus* virgin male adults of *Cyphoderris strepitans* (Leman et al., 2009). In the dragonfly *Paraphlebia zoe*, the females display a better NO production than males (Ruiz-Guzmán et al., 2013). The relationship between the degree of elaboration of secondary sexual characteristics and the strength of the immune response should be used by females as a reliable signal of the quality of the male (Zahavi, 1975), and JH has been proposed as the mechanism responsible for maintaining the reliability of this signal in insects (Rantala et al., 2003; Contreras-Garduño et al., 2009; Martínez-Lendech et al., 2019). Under this point of view, only the highest-quality males can elaborate extravagant attributes without becoming vulnerable to pathogens (Rantala et al., 2003; Contreras-Garduño et al., 2009). If the latter idea is correct, JH should impact, more importantly, the immune response of males than females because, in most species, males must signal their condition (Jacobs and Zuk, 2012). The current results partially support this hypothesis because more immune response parameters should be taken into account because Rantala et al. (2020)

showed that JH favored resistance against the fungus *Metarhizium robertsii* in males but not in females. The differential effect of JH might explain this last result according to pathogens, but also, more immune response parameters should be taken into account to know the contribution of JH according to pathogens considering the whole picture of the immune response. Our present and previous results (Rantala et al., 2020) suggest a complex effect of JH that depends on pathogens species and affects developmental stage (larvae *versus* adults) and sex.

Data

Data will be available upon request.

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CRediT authorship contribution statement

Tania Amaro-Sánchez: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Gloria Ruiz-Guzmán:** Methodology, Writing – review & editing. **Salvador Hernández-Martínez:** Methodology, Writing – review & editing. **Indrikis Krams:** Methodology, Writing – review & editing. **Markus J. Rantala:** Methodology, Writing – review & editing. **Jorge Contreras Garduño:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare no competing or financial interests.

Data availability

Data will be made available on request.

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