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RESEARCH REPORT

Are immune responses gender-related in *Carabus lefebvrei* (Coleoptera: Carabidae)?

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Abstract

The "live hard, die young" theory predicts the evolution of gender differences in immunocompetence, with males having a weaker immune system than females. To test this hypothesis in *Carabus lefebvrei*, total and basal phenoloxidase (PO) activities and lysozyme-like enzyme activity were compared among males and females of different reproductive status. The sexual dimorphism occurred only in reproductively active adults and for total and basal PO levels, while no significant differences were recorded between sexes in virgin adults. Differences were not recorded for lytic activity between sexes. Basal PO and lytic activities decreased in both males and females after mating, while the total PO value increased in males and decreased in females. Thus, resources seem to be invested to increase the humoral response in pre-reproductive phase forming a barrier against pathogens and preserving the fecundity and longevity of both sexes. Males preserve their survivorship in reproductive phase by increasing enzymatic levels in hemolymph to avoid fitness reduction due to the increased exposure to pathogen as result of mating. Females shift resources from PO and lytic activity to other physiological systems involved in reproduction in order to maximize their fitness.

Key Words: ecological immunology; life history; lytic activity; phenoloxidase; sexual dimorphism

Introduction

Studies on ecological immunology suggest that the variation and complexity in insect immune defence are closely related to different factors, intrinsic and extrinsic (Schmid-Hempel, 2003, 2005; Schmid-Hempel and Ebert, 2003; Sadd and Schmid-Hempel, 2009; Schulenburg *et al.*, 2009). Intrinsic factors such as genetic and physiological trade-offs and extrinsic ecological factors affect the expression of immune effectors and result in a specie-specific immune strategy to resist or tolerate pathogens (Zuk and Stoehr, 2002; Schmid-Hempel, 2003; Schmid-Hempel and Ebert, 2003; Schmid-Hempel, 2005) Sadd and Schmid-Hempel, 2009). Moreover, immune function is costly in term of evolving, maintaining and utilizing an immune effector system and trade-offs exist between immune defences and life history traits as reproduction, growth and development that share common resources and contribute to an animal's fitness (Zuk and Stoehr, 2002; Rolff and Siva-Jothy, 2003). Organisms should thus optimize immune defence through the life cycle often according to their age and gender. Females and males have different evolutionary challenges and they differ in a number of life-history traits. Therefore, a sexual dimorphism appear in their investment in immune defence such as have been investigated in a wide number of insect species including Coleoptera (Córdoba-Aguilar *et al.*, 2009; Shi and Sun, 2010), Diptera (McKean and Nunney, 2001; Ahmed *et al* 2002; Schwarzenbach *et al.,* 2005; Winterhalter and Fedorka, 2009; Vincent and Sharp, 2015), Hymenoptera (Cappa *et al.,* 2015), Lepidoptera (Meylaers *et al*., 2007; Stoehr, 2007; Lindsey and Altizer, 2009), Odonata (Rolff, 2001) and Orthoptera (Adamo *et al.*, 2001; Adamo, 2004; Pinera *et al.,* 2013; Galicia *et al*., 2014).

Actually, sexual selection is predicted to be an important evolutionary force affecting the evolution of the dimorphism in immunocompetence (Zuk and Stoehr, 2002, Schmid-Hempel, 2003, 2005; McKean and Nunney, 2008; Nunn *et al.,* 2009; Zuk, 2009). Forstad and Karter (1992) outlined potential costs of parasite-resistance in terms of the evolutionary

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process of sexual selection (known as the immunocompetence handicap hypothesis). The sexual dimorphic set of disease-resistance mechanisms have been demonstrated to be depended on environmental variations in terms of fitness-limiting resource availability (McKean and Nunney, 2005) and related to differences between males and females in resource allocation for self-maintenance (Stoehr and Kokko; 2006). Immune defences may be costly for both sexes and parasites may negatively affect male mating success and female fecundity or survival for both sexes (Rolff, 2002; Zuk and Stoehr, 2002; Stoehr and Kokko, 2006; Zuk, 2009). Based on Bateman's principle, male fitness is limited by access to females whereas female fitness is limited by offspring production and variation among male fitness is higher than among females. Males are thus generally expected to have a "live hard, die young" strategy and increase their fitness by increasing successful mating events, while females invest in immune response to favour longevity (Zuk and Stoehr, 2002; Zuk, 2009). However, males can invest more in immune defence than females in weak or absent sexual selection if the costs of infection to male mating success are high enough (Stoehr and Kokko, 2006).

Insect immunity involves the expression of a large array of cellular and humoral effectors to recognize and immobilize pathogens (Gillespie *et al.,* 1997; Ottaviani, 2005; Siva-Jothy *et al*., 2005). Humoral defences include the production of antimicrobial peptides (AMPs), reactive intermediates of oxygen or nitrogen and the prophenoloxidase enzymatic cascade (proPO) regulating melanization of haemolymph (Nappi and Ottaviani, 2000). The proPO-activating system comprises a complex cascade of serine proteases allowing the conversion of prophenoloxidase to PO (Marmaras *et al*., 1996; Gillespie *et al.*, 1997; Rolff *and* Siva-Jothy, 2003; Schmid-Hempel, 2005; Siva-Jothy *et al*. 2005). This enzymatic complex has been involved in physiological processes such as cuticular melanization and sclerotization and the defence reactions (wounding, clotting, melanotic incapsulation, production of cytotoxic molecules) (Marmaras *et al.*, 1996; Moreno-Garcia *et al.,* 2012). The main role of PO in melanogenesis is to convert phenols to quinones that subsequently polymerize to form melanin. In immune defence, natural activators of the proPO system are pathogen cell surface molecules such as β-1,3 glucans from fungal cell walls and lipopolysaccharides (LPS) and peptidoglycans from microbial cells. The melanin deposited onto the foreign target prevents the pathogen growth and reproduction and thus melanization is an important cell-mediated immune response in tissue repair and in pathogen sequestration (Söderhäll *et al*., 1994; Nappi and Vass, 2001; Cerenius and Söderhäll, 2004; Nappi and Christensen, 2005; González-Santoyo and Córdoba-Aguilar, 2012). AMPs such as lysozymes are present constitutively at a very low level in the hemolymph and their level increase upon challenge. The lysozymes belong to the c-type enzymes (Callewaert and Michiels, 2010) and perform a hydrolytic action against the peptidoglycan of Gram positive cell walls (Ratcliffe *et al.*, 1985; Gillespie *et al.*, 1997; Nappi and Ottaviani, 2000). They have also a low activity against Gram-negative bacteria (Yu *et al*., 2002) and a fungistatic property (Fiolka et al. 2005).

In the present study, the gender-specific and trade-off between immune defence and reproductive effort were tested using *Carabus lefebvrei*, as the model system. This is an Italian endemic carabid beetle that lives in beech, oak, chestnut and pine forests of the Central and Southern Apennines, from lower altitudes to about 1500 m a.s.l. It reproduces in spring, is active from April until September and hibernates as adults (Thiele, 1977; Turin *et al.,* 2003; Giglio *et al*., 2009). The habit of adults and larvae is typically that of a snail-eating predator and males display smaller than females (Turin *et al.*, 2003; Giglio *et al.*, 2012). Previous studies have shown different strategies in terms of cellular and humoral immune response to enhance the fitness of each life stage. The analysis demonstrated that the variation in speed and specificity of immune function across the developmental stages is correlated with differences in infection risk, life expectancy and biological function of the life cycle (Giglio *et al.*, 2008; Giglio and Giulianini, 2013).

In this study, we measured basal and total phenoloxidase (PO) enzyme activities and lysozyme-like enzyme activity as immunity markers involved in the pathogen resistance to evaluate the sexual dimorphism in *C. lefebvrei* immunocompetence. Laboratory tests were designed to compare virgin adults in their prereproductive phase with reproductive females and males after mating.

Material and Methods

Insect rearing and hemolymph collection

Carabus lefebvrei females and males were hand-collected in the Catena Costiera Mountains (39°19' N, 16°7' E, 900 - 1000 m a.s.l.; Southern Italy, Calabria) in early spring 2014. These adults are emerged in the early summer of previous year and hibernating under rotten pine barks in winder. In the laboratory, collected beetles were sexed and kept in groups (males and females) in 10 L plastic boxes, filled to a depth of 6.0 cm with moistened humus. The specimens were reared with a light regime of L/D = 15/9 h, 70 % relative humidity and a day/night room temperature of 23/18 °C. They were fed with snailsand daily observed until specimens show reproductive behaviour (mating events). After copulation, males were removed from the boxes to reduce the disturbance to females, which readily laid eggs. The eggs were transferred singly into 150 ml glass jars filled to a depth of 4.0 cm with moistened humus. Egg production and larval developmental time were recorded every two days until pupal instar and imago appearance. The haemolymph was collected from newly emerged adults 15-days-old (virgin females, n = 17 and males, n = 16) in their prereproductive phase and from reproductive females and males two days after mating events (see above) attesting their

reproductive status (n = 15 reproductive adults for both groups). The animals were CO_2 anesthetized before hemolymph collection. The hemolymph was collected by puncturing adults at the ventral level of the pro-mesothorax articulation with a 29-gauge needle. The first droplet of 10 µL of hemolymph was collected. Each hemolymph sample was immediately transferred into 190 µL ice-cold PBS (10mM sterile phosphate-buffered saline, Sigma-Aldrich) in a 1.5 mL eppendorf tube and centrifuged at 1700 g for 5 min at 4 °C. The cell-free hemolymph obtained as supernatant was collected and stored at -20 °C until enzymatic assays.

Phenoloxidase specificity, zymogen activation and specificity of reaction

For determining enzyme specificity and zymogen activation, the PO activity was measured spectrophotometrically according to Winder and Harris (1991), using L-DOPA as substrate and 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH, 6 mM) as a specific reagent. Briefly, 40 µL of hemolymph-buffer solution were incubated for 30 min at 20 °C with 40 µL of PBS (Na_2HPO_4 1M, KH_2PO_4 0,01 M; NaCl 1.5 M, pH 7.4) and 40 µL of DOPA-MBTH reaction mixture (20 mM L-DOPA and MBTH in distilled water). After the reaction, dopaquinone was detected within 60 min at 5 min intervals by spectrophotometric measurement at 505 nm. The PO activity was expressed as units (Us) for min, where one U was defined as the amount of enzyme required to produce an increase in the absorbance at 505 nm of 0.001 units min^{-1}. To check for specificity of the enzyme reaction, before L-DOPA and MBTH were added, the homogenate was incubated (20 min at 20 °C) with trypsin or with 1-phenyl-2-thiourea (PTU) in PBS at 1 mM final concentration. This inhibitor acts by chelating the copper at the active site, and it is known to be one of the most effective PO inhibitors (Kahn, 1985; Aspàn *et al*., 1995; Klabunde *et al*., 1998).

Basal and total phenoloxidase enzyme activity

Phenoloxidase (PO) activity was monitored spectrophotometrically as the formation of dopachrome from 3, 4-dihydroxy-L-phenylalanine (L-DOPA, Sigma-Aldrich). For determination of basal PO, 20 µL of haemolymph-buffer solution were taken and mixed with 180 µL of L-DOPA (3 mg/mL in PBS) in a microtiter plate. For determination of total PO enzyme activity, 30 µL of hemolymph -buffer solution were added to 30 µL of methanol that chemically activates PO from its inactive zymogen, prophenoloxidase (proPO). The hemolymph-methanol mixture was incubated for 5 min at room temperature and 20 µL were mixed with 180 µL of L-DOPA (3 mg/mL in PBS) in a microtiter plate. The basal and total phenoloxidase enzyme activity at 20 °C was recorded at 492 nm for 30 min in 5 min intervals using a plate reader (Sirio S, SEAC). All samples were assayed in duplicate. The enzyme activity was measured as the slope (absorbance vs time) of the reaction curve during the linear phase of the reaction (*Vmax* value; between 15 and 20 min for reproductive females and 0 and 5 min for all the others after the reaction began). The slope of the reaction curve at V_{max} was plotted as absorbance per µL of hemolymph per min for female and male of different mating status.

Lysozyme-like enzyme activity

A turbidometric assay was used to measure lysozyme-like activity in the hemocyte-free plasma. The assay is based upon the lyses of the lysozyme-sensitive Gram-positive bacterium *Micrococcus lysodeikticus*. 10 µL of hemolymph-PBS mixture (described above) was loaded into the well of a 96-well microplate followed by 190 µL of a *Micrococcus lysodeikticus* (strain ATCC 7468, DSMZ) cell wall suspension ($1.6x10^8$ cell/ml of cold PBS). The reductions in turbidity in the wells were read on a plate reader (Sirio S, SEAC) at 25 °C for 45 min in 5 min intervals at 450 nm. All samples were assayed in duplicate. The enzyme activity was reported as the change in absorbance (absorbance vs time) of the reaction curve during the linear phase of the reaction (V_{max} value; between 5 and 15 min after the reaction began). The slope of the reaction curve at V_{max} was plotted as absorbance per µL of hemolymph per min, for female and male samples at different mating status. Standards of enzyme activity were made using lysozyme from chicken egg whites (Sigma) and a suspension of *M. lysodeikticus* as substrate. The standards were run simultaneous with the hemolymph samples to confirm that assay was progressing as expected (*i.e*., absorbance values decreasing).

Hemolymph protein content

Protein content was estimated by the method of Bradford (Bradford, 1976) with bovine serum albumin (BSA) as a standard using 100 µl of hemolymph-buffer solution for each sample and 900 µl of Bradford reagent (Sigma-Aldrich).

Statistical analyses

Statistical analyses were performed using R version 3.0.1 software (R Development Core Team 2013). Total and basal PO enzyme activities, lysozyme-like enzyme activity and hemolymph protein content were measured and compared among reproductive female and male samples as well as virgin ones. The differences among Vmax were assessed by nonparametric statistics, *i.e*., Kruskal-Wallis rank sum test (KWT) followed by post-hoc Wilcoxon rank sum test pairwise comparisons with Bonferroni correction (PWT), since the null hypothesis of the Bartlett test could not be rejected. The box and whisker plots were drawn with the boxplot command. All values are reported as mean ± SE in the text.

Results

Phenoloxidase specificity, zymogen activation and specificity of reaction

A lower PO activity (males 8.98 ± 0.90 U, females 7.46 ± 0.65; n = 5) was found when the samples were assayed in the absence of activating enzyme treatment, whereas it was raised by trypsin (males 11.41 ± 0.94 U, females 9.03 ± 0.91 U; n =

5). The specificity of the PO reaction was demonstrated by the specific inhibitor phenylthiourea added to homogenates before the activation with trypsin. PO activity of samples, compared to untreated or activated controls, was lowered (males 3.54 ± 0.66 U; females 1.25 ± 0.34 U).

Basal and total phenoloxidase enzyme activity

Highly significant differences were detected in total (KWT, $p < 0.0001$) (Fig. 1A) and basal (KWT, $p < 0.0001$) (Fig. 1B) PO activity of *C. lefebvrei* among females and males of different reproductive status. In females, there were no significant differences in the total PO activity (PWT, $p = 0.062$), while the basal PO activity decreased significantly in reproductive specimens (PWT, $p = 0.002$). In males, the total PO activity was significantly lower in virgin than reproductive ones (PWT, $p < 0.0001$), whilst a contrary trend was recorded in basal PO activity (PWT, $p = 0.014$). In reproductive adults, the total PO activity was significantly higher in males than females (PWT, $p < 0.0001$) as well as the basal PO activity (PWT, $p < 0.0001$).

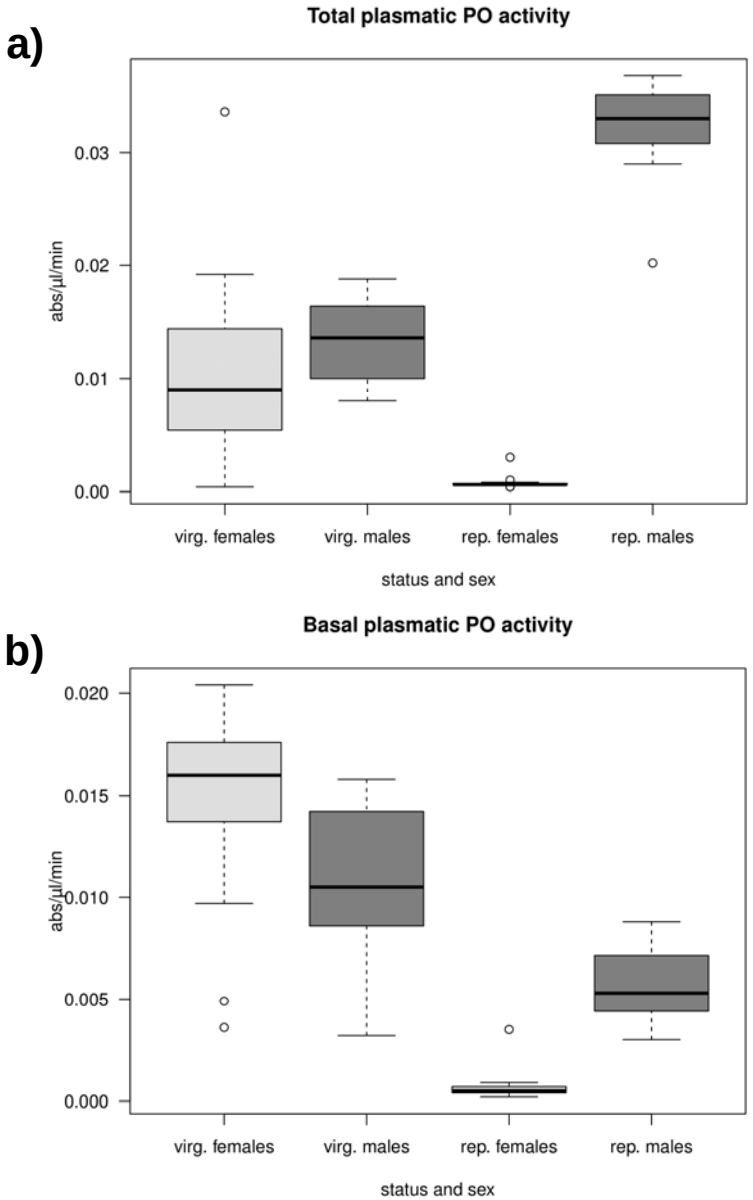

Fig. 1 Total a) and basal b) phenoloxidase activity in *C. lefebvrei* measured as the slope of the reaction curve at Vmax. The enzymatic activity of reproductive and virgin females and males was recorded as absorbance units for µL of hemolymph per min (for statistics see the text). The boxplot represents the interquartile range (IQR = Q3-Q1) and bars represent first (Q1, top) and third quartiles (Q3, bottom) of phenoloxidase activity values from reproductive females and males and in virgin ones. The central horizontal black line indicates the median. The ends of dashed lines (ends of the whiskers) represent the lowest datum and the highest datum.

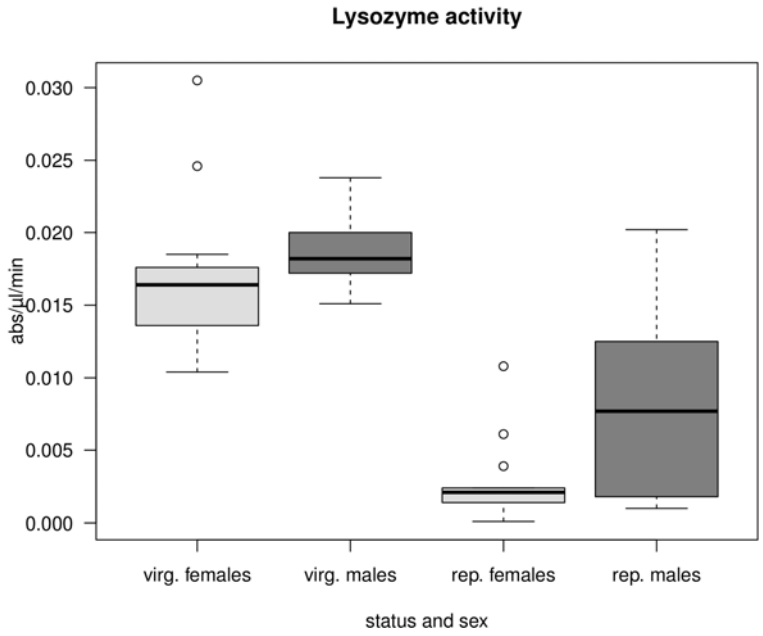

Fig. 2 Lysozyme-like enzyme activity in *C. lefebvrei* measured as the slope of the reaction curve at Vmax. The lytic activity in reproductive and virgin females and males was recorded as units for µL of hemolymph per min (for statistics see the text). The boxplot represents the interquartile range (IQR = Q3-Q1) and bars represent first (Q1, top) and third quartiles (Q3, bottom) of phenoloxidase activity values from reproductive females and males and in virgin ones. The central horizontal black line indicates the median. The ends of dashed lines (ends of the whiskers) represent the lowest datum and the highest datum.

In virgin adults, there were no significant differences in the basal (PWT, p = 0.164) and total (PWT, p = 1.0) PO activities between males and females.

Lysozyme-like enzyme activity in the hemolymph plasma

The results showed a significant difference in baseline lytic activity (KWT, p < 0.0001) (Fig. 2) among females and males at different reproductive status. The enzymatic activity decreases significantly in both females (PWT, p < 0.0001) and males (PWT, p < 0.0001) at reproductive status. There were no significant differences between sexes in both reproductive (PWT, p = 0.370) and virgin adults (PWT, p = 0.349).

Hemolymph protein content

The total protein content was recorded in hemocyte-free plasma of both males and female at different reproductive status and significant differences were detected (KWT, p < 0.0001) (Fig. 3). There was no significant differences in plasmatic protein content between reproductive males and females (PWT, p = 0.163) and virgin males and females (PWT, p = 1.0). However, there was a highly significant increase in protein content in reproductive adults for both females (PWT, p < 0.0001) and males (PWT, p = < 0.0001) compared to virgins.

Discussion

Previous studies on dimorphism of immune response have investigated optimal allocation of resources between immunity, survival and reproduction in males and females (Stoehr and Kokko, 2006). The outline of defence strategy may vary generally under environmental selective pressure as a result of a wide range of factors (ecological, genetic and evolutionary) closely related to different life histories for males and females (Viney *et al*. 2005; Stoehr and Kokko, 2006; Restif and Amos, 2010). In our study, reproductive males of *C. lefebvrei* showed high values of basal and total PO enzyme activities compared with females after mating, while enzymatic activities did not differ between sexes in virgin adults. In spite of that, there was no sexual dimorphism in baseline lysozyme-like enzyme activity among females and males at different mating status. Some studies have formerly found that the direction and magnitude of sex differences in immune-competence could be different for each component of immune defence (Zuk and Stoehr, 2002; Stoehr and Kokko, 2006; Stoehr, 2007) and limited mainly by resource availability (McKean and Nunney, 2005; Galicia *et al.*, 2014). In some species there is strong evidence that males and females may emphasize different immune components in relation to age, mating, sexual antagonism and attractiveness or food

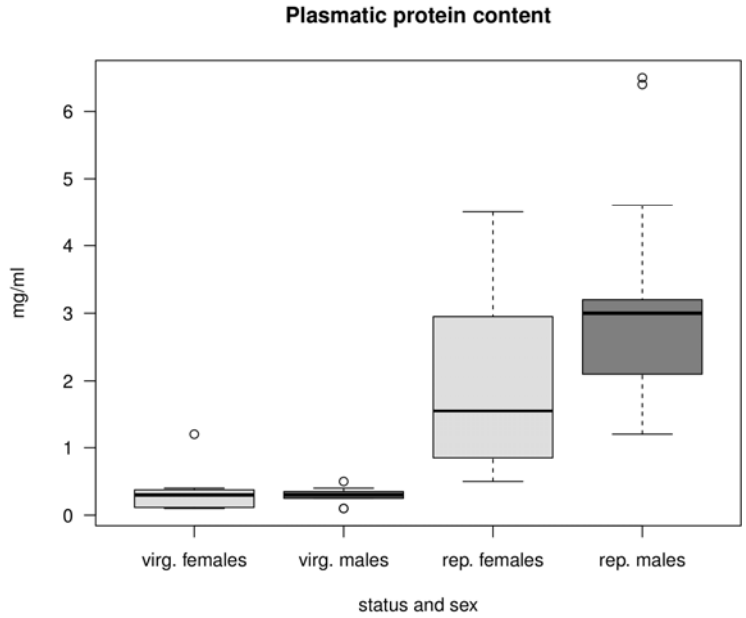

Fig. 3 Protein content in *C. lefebvrei* cell-free hemolymph for reproductive and virgin females and males (for statistics see the text). The boxplot represents the interquartile range (IQR = Q3-Q1) and bars represent first (Q1, top) and third quartiles (Q3, bottom) of phenoloxidase activity values from reproductive females and males and in virgin ones. The central horizontal black line indicates the median. The ends of dashed lines (ends of the whiskers) represent the lowest datum and the highest) datum.

availability (Adamo, 2001; Lawniczak *et al*., 2006; Stoehr, 2007; Córdoba-Aguilar *et al.*, 2009; Winterhalter and Fedorka, 2009; Kivleniece *et al.,* 2010; Galicia *et al.*, 2014; Vincent and Gwynne, 2014; Vincent and Sharp, 2014).

Our results show that the investment strategy in immunocompetence varies with reproductive status in *C. lefebvrei* adults, but further investigation is needed to clarify if change in immune responses may be often age-dependent for both sexes and if male may have a limited amount of resources to invest optimally in both the immune system and sexual attractiveness. We assume that the sexual dimorphism of PO enzymatic activity in *C. lefebvrei* adults are due to a different strategy to balance for resource allocation between physiological and ecological costs in both sexes. Moreover, the significant increase of hemolymph protein content was positively correlated with the reproductive status of adults and confirmed the specificity of enzymatic activities recorded in absence of infection. To exclude that enzymes with similar activity such as peroxidases, laccases and catalases are involved in recorded enzymatic activity, the proPO activating system were characterized and displayed the Calcium-independent PO activity enhanced by trypsin (Cerenius and Söderhall, 2004). Phenylthiourea, a specific PO inhibitor, supported the reaction specificity with an inhibition of 86 % of activity in females and 68 % in males.

Some studies have provided evidence for a reduction of immune function as a result of increased reproductive activity in absence of infection (Rolff and Siva-Jothy, 2002; Fedorka *et al*., 2004; Otti, 2015). However, natural selection may favour immunity if the infection pressure is high (Schwenke *et al*., 2016). *C. lefebvrei* males gain fitness by investing heavily in immunity to protect themselves from an exposure to parasites due to their larger number of mating events. Females mating one time have a reduced exposure risks compared to males. As a result, further investigation will be addressed to verify that reproductive females decrease the immune response because they shift resources from proPO system and lytic hemolymphatic activity to other physiological systems involved in reproduction including production of eggs, reception and storage of sperm, fertilization and oviposition.

High levels of both basal PO and lysozyme-like enzyme activities recorded in virgin adults are likely to preserve the adult survivorship against infections until the reproductive phase. Actually, the available data strengthen the leading role phenoloxidase and lysozyme-like enzyme activities play in disease resistance (Adamo, 2004). Studies on cricket that have estimated disease resistance based on sex differences have shown that lysozyme-like activity is maintained at a low level and increases in response to bacterial challenge (Adamo, 2004; Piñera *et al.*, 2013; Galicia *et al.*, 2014). In many species, the degree of cuticular melanization and wound repair is a strong indicator of resistance to pathogens and it is correlated with PO activity in the cuticle, hemolymph and midgut (Barnes and Siva-Jothy, 2000; Wilson *et al.,* 2001). In *C. lefebvrei,* as the cuticle does not offer full protection before its complete melanization, basal PO expression may be up-regulated to melanize the cuticle mainly in

newly emerging adults which are more exposed to wounds and thus more susceptible to infections. At the same time, this species shows an increase in predation activity before mating to accumulate metabolic resources so, as a result, it is exposed to potential infectious microorganisms via oral consumption of prey (snails). Thus, the high level of lytic hemolymphatic activity in pre-reproductive phase of adulthood might increase humoral defence to maintain an effective protection increasing the organism's fitness.

In conclusion, here we found that two different immune effector systems, PO activity and lytic activity, show variation over the lifetime of *C. lefebvrei* males and females. The sexual dimorphism was recorded for PO activity, but not for lytic activity. This shows that measuring a single component of the immune system in not going to provide an overall indicator of immunocompetence. Furthermore, *C. lefebvrei* reproductive males have higher values of both PO and lytic activity than reproductive females. This result confirms that immune function is not a simple, static process, but rather a dynamic system of interrelated mechanisms that are differentially effective and may not be generalizable among species. Immunity and the ability to reproduce are closely related in order to maximize the fitness of each species (Schmid-Hempel, 2003; Schulenburg *et al*., 2009) and sexual differences in immune investment are difficult to predict. At the last, lower enzymatic activity in reproductive females demonstrates that the investment of high energies amount to immunocompetence is not always the best choice in term of fitness.

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