ISJ 14: 165-173, 2017 ISSN 1824-307X

RESEARCH REPORT

Gender-related variations in hemolymph parameters of *Carabus lefebvrei* (Coleoptera: Carabidae): HPLC analysis and phenoloxidase activity

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Accepted April 26, 2017

Abstract

We characterized the enzymatic activity of basal and total phenoloxidase and HLPC and SDS-PAGE profiles in hemolymph of *Carabus lefebvrei* males and females at different reproductive status. The phenoloxidase activity was activated by trypsin and inhibited by phenoloxidase activity specific inhibitor phenylthiourea. Our results demonstrated that both in males and females, there were no significant differences in the basal phenoloxidase activity between reproductive and virgin beetles, while the total phenoloxidase activity increased significantly in virgin specimens. 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. The hemolymph DOPA-MBTH assay on polyacrilamide gel electrophoresis showed a high activity of monomeric form with an apparent molecular weight of 90 kDa and a dimer of about 170 kDa, also multimeric bands were present in both sexes. In the SDS-PAGE general protein pattern, specific bands were evident for reproductive and virgin males and females as biochemical markers of sexual difference in immunocompetence. Reproducible differences in peaks were recorded in HPLC analysis performed on virgin and reproductive males and females

Key Words: ecological immunology; life history; HPLC; phenoloxidase; sexual dimorphism; SDS-PAGE

Introduction

The gender-related differences in immunological competency have become of great interest at both physiological and evolutionary level. Previous studies on dimorphism of immune response have investigated optimal allocation of resources between immunity, survival and reproduction in males and females (Sequeira *et al.*, 1995; Kurtz *et al.*, 2000; Cheng and Chen, 2001; Stoehr and Kokko, 2006; Calleri *et al.*, 2007; McKean and Nunney, 2005, 2008; Nunn *et al.,* 2009; Matozzo and Marin, 2010; Schwenke *et al.*, 2016). 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 (Rolff, 2002; Viney *et al.*, 2005; Stoehr and Kokko, 2006; Zuk, 2009; Restif and Amos, 2010). Reproduction, growth, development and immune responses contribute to an animal's fitness and the trade-off between them is likely due to alternative allocation of limiting energetic resources (Zuk and Stoehr, 2002; Rolff and Siva-Jothy, 2003; Schmid-Hempel, 2003; Sadd and Schmid-Hempel, 2009; Vincent and Gwynne, 2014). It is important to appreciate that plastic physiological trade-offs between reproduction and immune competence are mainly a matter of optimization rather than maximization to preserve the individual survival. Studies on insect immunity have measured a number of immune effectors related to both cellular and humoral reactions, focusing on defence variation in both evolutionary and ecological context (Gillespie *et al.,* 1997; Nappi and Ottaviani, 2000; Schmid-Hempel and Ebert, 2003; Schmid-Hempel, 2003, 2005; Siva-Jothy *et al.*, 2005; Ottaviani, 2005). The background levels of these traits in both sexes can be measured in the absence of infection (constitutive immunity) (Rantala and Kortet, 2003) or after presenting an immune elicitor (induced immunity) (Adamo, 2004;

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Galicia *et al*., 2014). Immune traits that are commonly measured include survivorship of pathogenic infection, count or activity of circulating haemocytes, antimicrobial activity, encapsulation and phenoloxidase activity (Ratcliffe *et al.,* 1985; Adamo *et al.*, 2001, 2004; McKean and Nunney, 2001; Rolff, 2001; Ahmed *et al.,* 2002; Schwarzenbach *et al.,* 2005; Meylaers *et al*., 2007; Stoehr, 2007; Córdoba-Aguilar *et al.*, 2009; Lindsey and Altizer, 2009; Winterhalter and Fedorka, 2009; Shi and Sun, 2010; Piñera *et al.,* 2013; Galicia *et al*., 2014; Vincent and Sharp, 2014; Cappa *et al.,* 2015).

The prophenoloxidase-activating (proPO) system comprises a complex cascade of serine proteases allowing the conversion of prophenoloxidase to phenoloxidase (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 the 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).

We recently demonstrated that *Carabus lefebvrei* is a good new model system to test on the one hand immune strategies to enhance the fitness of each life stage (Giglio *et al.*, 2008; Giglio and Giulianini, 2013; Giglio *et al.*, 2016) and on the other hand gender-specific immune responses for PO and lysozyme-like enzyme activities (Giglio *et al.,* 2016). *C. lefebvrei* 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 are smaller than females (Turin *et al.*, 2003; Giglio *et al.*, 2012).

In this study, to add new information, we characterized the specificity of proPO enzymatic activity and HLPC and SDS-PAGE profiles of hemolymph fractions as immunity marker to evaluate the difference between males and females in *C. lefebvrei* immunocompetence. Laboratory tests were designed to compare virgin adults in their pre-reproductive phase with reproductive females and males after mating.

Material and Methods

Insect rearing and hemolymph collection

C. 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 winter. In the laboratory, beetles were sexed looking at the copulatory apparatus and divided in groups (males and females) in 10 L plastic boxes, filled with 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 snails (*Helix* sp.) and 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 hemolymph was collected from newly emerged adults 15-days-old (virgin females, n = 17 and males, n = 16) in their pre reproductive 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 1,700*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 3,4-dihydroxy-L-phenylalanine (L-DOPA, Sigma-Aldrich) 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 increases in the absorbance at 505 nm of 0.001 unit min-1 for mg of protein. Phenoloxidase

Table 1 Phenoloxidase activity of cell-free hemolymph in reproductive and virgin males and females of *C. lefebvrei*

Treatments	*Phenoloxidase activity*			
	Reproductive		Virgin	
	Male	Female	Male	Female
controls	8.98 ± 0.8	7.46 ± 0.65	4.51 ± 2.8 b	5.49 ± 1.2 b
Trypsin (0.5mg/ml)	11.41 ± 0.9 abc	9.03 ± 0.91 abc	18.6 ± 6.6 ab	20.6 ± 2.2 ab
PO inhibitors				
PTU (1 mM)	3.54 ± 0.66 a	1.25 ± 0.34 a	1.2 ± 5.3 a	1.2 ± 2.2 a
DETC (10 mM)	2.53 ± 0.84 a	3.63 ± 0.54 a	1.1 ± 3.1 a	1.2 ± 1.3 a

The enzymatic activity of control (basal PO), trypsin treated-hemolymph (total PO) and hemolymph incubated for 20 min with PO inhibitor was expressed as U/min/mg protein.
Values are the mean of six experiments performed in triplicate ± SD.
Means (±SD) followed by the same letter are significantly different a: respect to the control; b respect to the opposite reproductive stage; c: respect to the opposite reproductive sex (Tukey t test $p < 0.05$).

activity in hemolymph was detected without (control) and with the addiction of trypsin, which enzymatically actives PO from its inactive zymogen, pro-PO. We thus measured basal (control) and total PO activity in both female and male samples at different reproductive status To check for specificity of the enzyme reaction, before L-DOPA and MBTH were added, the hemolymph-buffer solution was incubated (20 min at 20 °C) with trypsin or with the 1-phenyl-2-thiourea (PTU) or diethilthiocarbamate (DETC) 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 inhibitor (Kahn, 1985; Aspàn *et al*., 1995; Klabunde *et al*., 1998).

Electrophoresis and PO activity of haemolymph

PO activity was assessed by polyacrylamide gel as described by Cardenas and Dankert (2000) with some modification. The haemolymphs from reproductive and virgin females and males showing PO activity, were subjected to electrophoresis in polyacrylamide gel performed according to the method of Laemmli (1970) using a Mini Protean II cell (Bio-Rad). The gels were calibrated with high molecular weight range standard protein (Sigma-Aldrich (USA). To identify the PO activity of the protein bands, the gels were washed twice with PBS-T (NaCl 0.1 M; KCl 0.02 M; KH_2PO_4 0.01 M; Na_2HPO_4 0.06 M, pH 7.4, 2.5 % di Triton X-100), and a final wash of 10 min. in PBS 1x without Triton X-100. The gel was incubated in a solution containing L-DOPA 20 mM and MBTH 6 mM in distilled water. After 1 h of incubation, the gel was washed several times in distilled water. In relation to the regression curve obtained by means of the software Alpha Ease FC molecular weights of the obtained bands were calculated. SDS-PAGE (15 %, unless otherwise indicated) was performed also according to the method of Laemmli (1970) and proteins were stained with Coomassie Blue. Gels were calibrated with molecular mass markers (SigmaMarker low range 6,5 - 66 kDa, Sigma-Aldrich), and the calculated kDa was the average ± SD of 6 distinct analyses. Prior to electrophoresis, samples were boiled for 5 min in a sample buffer containing 5 % β-2-mecaptoethanol as reducing agent, unless otherwise indicated. Software Alpha Ease FC Stand-alone V.4.0 (Spot Density tools) was used to calculate the integrated density value (IDV: sum of all the pixel values after background correction/area) of the stained protein bands.

HPLC size exclusion chromatography

The hemolymph from both reproductive and virgin females and males was subjected to HPLC separation Silica Column C18 interchrom UPSODB-25QS 250X4.6mm on a liquid chromatography HPLC system (Shimadzu Scientific Instruments, SSI, North America). Column was washed with TBS (NaCl 150 mM, TRIS HCl 10 mM, pH 7.4). An injection volume of 200 µL was used at a flow rate of 1 mL/min for 30 min. The chromatogram was recorded with a UV detector at 280 nm (mAU) in TBS. The eluate corresponding to each peak was collected in 1 mL/min fractions. The collected fractions were concentrated by centrifugation at 500*g* with micro-concentrators (3K Omega Centrifugal Devices Nanosep), and the final concentrated samples were stored at -80 °C until use.

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

PO enzyme activities were measured and compared among reproductive female and male

Table 2 Statistical significant summarising the comparison results of Tukey t test of phenoloxidase activity of cell-free hemolymph in reproductive (RM) and virgin males (VM) and females (RF and VF) of *C. lefebvrei*

		CONTROL (Basal PO)				*TRYPSIN (Total PO)*			
		RM	RF	VM	VF	RMT	RFT	VMT	VFT
CONTROL	RM		NSS	$p < 0.05$	NSS	$p < 0.05$	NSS	$p < 0.05$	$p < 0.05$
(Basal PO)	RF				$p < 0.05$	NSS	$p < 0.05$	$p < 0.05$	$p < 0.05$
	VM					$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
	VF					$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
TRYPSIN	RMT						NSS	$p < 0.05$	$p < 0.05$
(Total PO)	RFT							NSS	$p < 0.05$
	VMT								NSS

The enzymatic activity of control (basal PO), trypsin treated-hemolymph (total PO).

samples as well as virgin ones. Student's t-test was used to estimate statistical significance. Multiple comparisons were performed with one-way analysis of variance (ANOVA), and different groups were compared by using Tukey's t-test. The software package STATISTICA 5.5 (StatSoft, Tulsa, OK, USA) was used for statistical analyses. Standard deviations were calculated on four experiments. p-value ≤ 0.05 was considered statistically significant.

Results

Characterization of phenoloxidase activity and zymogen activation

A lower PO activity was found when the samples were assayed in the absence of activating enzyme treatment (control, Table 1). Whereas the enzymatic activity increased significantly when samples were incubated with trypsin indicating the existence of inducible proenzyme.

The specificity of the PO reaction was demonstrated in both females and males at different reproductive status by the specific inhibitor PTU and DETC added to hemolymph-buffer solution before the activation with trypsin. PO activity, after inhibition with PTU and DETC, compared to untreated (control) or trypsin activated samples, was lowered (Tables 1, 2). The residual enzymatic activity could be attributable to the other class of enzymes like laccases or peroxidases.

A significant increase of the total PO activity in trypsin activated samples were detected for females and males of *C. lefebvrei* at the different reproductive status (Tables 1, 2). Moreover, there were no significant differences in the basal PO activity between reproductive and virgin adults, while the total PO activity increased significantly in virgin specimens ($p \leq 0.05$).

SDS PAGE and PO activity

The SDS PAGE are conducted with the same total protein concentration to evaluate differences and similarity among reproductive and virgin females and males. Specific bands of an apparent molecular weight of 90 and 105 kDa clear mark the difference between virgin and reproductive males and females (Fig. 1, lanes 1 and 4 arrows). Another specific band of an apparent molecular weight of 45 kDa seems to be specific for the adult females (Figs 1A, C).

The protein size analysis and DOPA-MBTH assay, disclosed two active proteins with an apparent molecular weight of 90.0 and 170.0 kDa at different concentrations, whereas, due to the SDS activating effect on the proenzyme, the proPO activation process could not be shown, whereas modulation of an oligomerization process can be suggested (Fig. 1B).

High pressure liquid chromatography analysis

The chromatographic profiles of reproductive males and females displayed several confluent peaks in term of elution time and concentrations except for peaks indicated by arrows (Fig. 2A). Peaks marked by arrows at 3.5, 3.75 and 5.75 min/mL showed higher concentration in reproductive females than males. Whereas, at 11.5 and 18 min/ml elution time peaks showed that the concentration is lower in reproductive females than males.

The chromatographic profiles of hemolymph for virgin male and female were overlapped (Fig. 2B). During the 40 min of profile separation running, several different peaks are revealed but only one peak at 7.25 min/mL of elution time showed a very high different concentration in virgin female compared with males.

In females, an overlapped of comparative chromatographic profiles in reproductive and virgin females was recorded (Fig. 3A). Several common peaks with similar amplitude were identified between reproductive and virgin but with one very clear difference in concentration for the peak at 7.25 min/mL of elution time in virgin females. In males, three additional peaks respectively at 11.75, 18 and 21.5 min/mL seem to be characteristic of virgin males compared with reproductive ones (Fig. 3B).

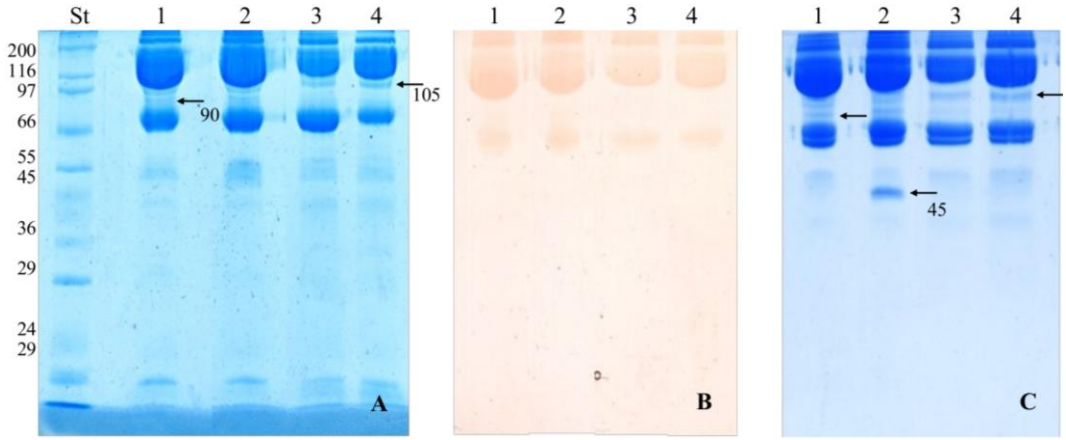

Fig. 1 Electrophoretic patterns of the hemolymph show male and female samples at different reproductive status performed with SDS-PAGE/Coomassie blueunder reducing conditions (A), SDS-PAGE-DOPA-MBTH in non-reducing conditions (B) and SDS-PAGE/Coomassie blue in non-reducing conditions (C). The arrows indicate specific bands that clearly show the difference between virgin and reproductive males and females. St: standard; Lane 1: reproductive males; Lane 2: reproductive females; Lane 3: virgin males; Lane 4: virgin females.

Discussion

The gender-related immunomodulation could be strategic for physiology, adaptation and evolution of animals. Previous study on *C. lefebvrei* showed that gender differences occur only for PO activity that was significantly higher in reproductive males than females but not for lysozyme-like enzyme activity (Giglio *et al.,* 2016). To exclude that other enzymes with similar activity (peroxidases, laccases and catalases) are involved in the reaction of the enzymatic activity, we characterized and displayed Calcium-independent PO activity enhanced by trypsin. Phenylthiourea and DETC, a specific PO inhibitor, supported the reaction specificity and trypsin treatment significantly enhanced. Our data on PO activity, protein contents and HPLC analysis confirmed that the investment strategy in immunocompetence of *C. lefebvrei* adults varies with reproductive status to balance for resource allocation between physiological and ecological costs in both sexes.

After pro-PO activation with trypsin, virgin females and males of *C. lefebvrei* showed higher values of total PO enzyme activities compared with reproductive adults, while basal PO enzymatic activities was significantly higher in reproductive ones. The higher level of pro-PO enzyme in virgin adults confirms that the inducible PO activity is likely to preserve the adult survivorship against infections until the reproductive phase to maintain an effective protection increasing the organism's fitness. Furthermore, the reduction of immune function in reproductive adults is a result of increased reproductive activity in absence of infection (Rolff and Siva-Jothy, 2002; Fedorka *et al.*, 2004; Otti, 2015).

The protein content in hemolymph of the reproductive males and females were found to be about five time more concentrated than virgin status. The protein content from the hemolymph subjected to SDS-PAGE and stained with Coomassie blue shows two specific bands with apparent molecular weight of 90 and 105 kDa that clear mark the difference between virgin and reproductive adults. Another specific band with an apparent molecular weight of 45 kDa seem to be specific for reproductive females. The same band stained with DOPA-MBTH assay on polyacrilamide gel electrophoresis shows a canonical high concentration of monomeric/dimeric form showing an high activity, also multimeric form are present both in reproductive and virgin males and females.

The overlapping of the chromatographic profiles of males and females sample at different reproductive status highlights that the haemolymph of reproductive and virgin females present the highest peaks in term of concentration, with the exception of the peak at 11.5 min/mL in both status and that 18.0 min/mL for reproductive status. The overlapping of the chromatographic profiles of reproductive and virgin females males shows in both cases the highest peaks for virgin status. This first finding strengthened the evidence that on the one hand 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). One the other hand, males and females may emphasize different immune components in relation to age, mating, sexual antagonism and attractiveness or food 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). *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. The high level of activable

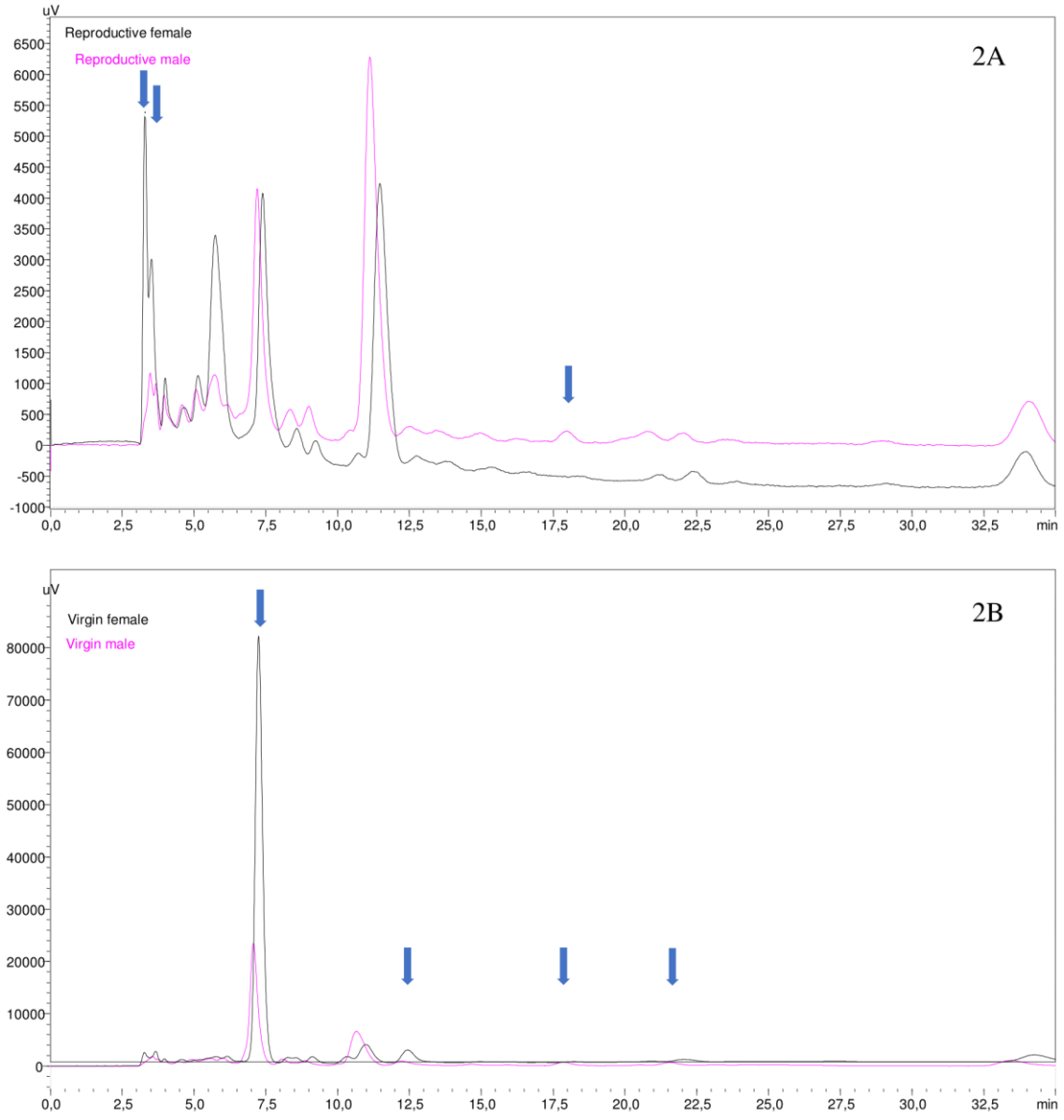

Fig. 2 HPLC separation of the hemolymph from both female and male at different reproductive status using Silica Column C18 interchrom UPSODB-25QS 250X4.6mm on a liquid chromatography HPLC system. Elution was performed with TBS over 40 min at a flow rate of 1 ml/min. Absorbance peaks were monitored at 280 nm. A) Overlapping of the chromatographic profiles of reproductive males and females. B) Overlapping of the chromatographic profiles of virgin males and females. The arrows indicate the different concentration of the confluent peaks of males and females.

PO proenzyme present in the virgin males and females support the hypothesis that in reproductive females and males the immune response decrease because they shift resources from proPO hemolymphatic activity to other physiological systems involved in reproduction including production of eggs, reception and storage of sperm, fertilization and oviposition.

In conclusion, here we found that the PO activity and protein composition, show variation over the lifetime of *C. lefebvrei* males and females. The sexual dimorphism was recorded for PO activity and marked by specific band in PAGE and HPLC analyses. 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 and the investment of high energies amount to immunocompetence is not always the best choice in term of fitness.

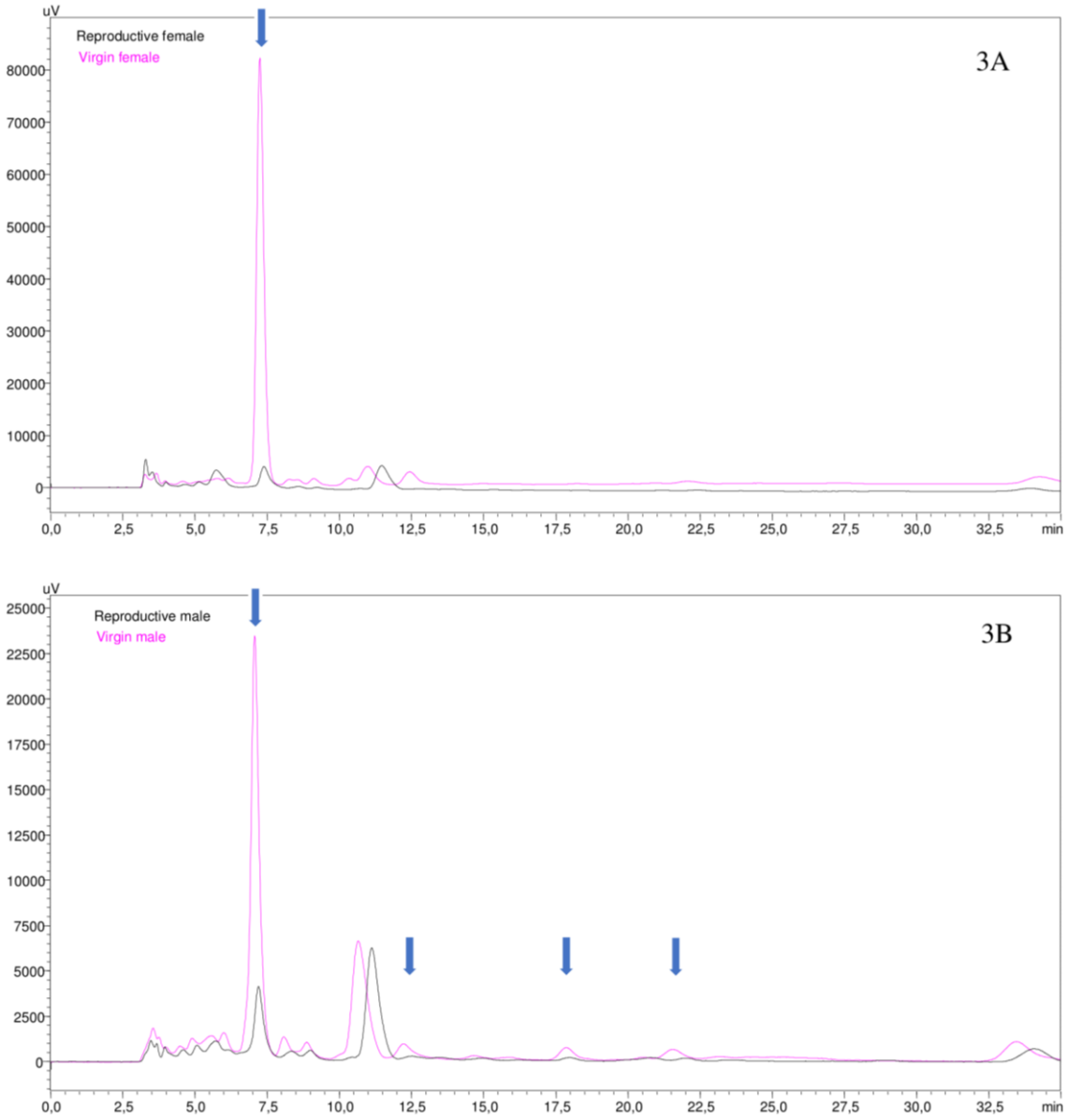

Fig. 3 HPLC separation of the hemolymph from both female and male at different reproductive status using Silica Column C18 interchrom UPSODB-25QS 250X4.6mm on a liquid chromatography HPLC system. Elution was performed with TBS over 40 min at a flow rate of 1 ml/min. Absorbance peaks were monitored at 280 nm. A) Overlapping of the chromatographic profiles of reproductive and virgin females. B) Overlapping of the chromatographic profiles of reproductive and virgin males. The arrows indicate the different concentration of the confluent peaks of males and females.

Acknowledgements

This work was supported by the grant (n° A.001.2014.EX60) assigned to Giglio A from the Ministry of Education, University and Research (MIUR) and grant from the Italian Ministry of Education (PRIN 2010-2011 to Cammarata M), co-funded by the University of Palermo, Italy.

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