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Vitellogenin and Lipovitellin from the prawn *Macrobrachium borellii* as hydrocarbon pollution biomarker

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ABSTRACT

During reproduction vitellogenin (VTG) is transported to vitellogenic oocytes as a precursor of egg yolk lipovitellin (LV). As VTG synthesis is affected by environmental stressors, it is widely used as biomarker in endocrine disruption studies. However, it has seldom been employed to evaluate invertebrate hydrocarbon pollution. An ELISA with anti-LV antibody was developed to evaluate the impact of water-soluble fraction of crude oil (WSF) on *Macrobrachium borellii* vitellogenesis.

Prawn VTG concentration was within the range reported for other crustaceans; LV values were positively correlated with gonadosomatic index (GSI). Females at different vitellogenic stages were exposed to a sub-lethal concentration of WSF for 7 days. Exposed animals with GSI > 7 increased their VTG and LV titer as compared to control organisms (190% and 140%, respectively). VTG levels in *M. borellii* were upregulated and highly sensitive to WSF exposure. This assay could be employed as a biomarker for freshwater hydrocarbon pollution.

1. Introduction

Among the different types of environmental pollution, the impact of anthropogenic hydrocarbons in aquatic and terrestrial ecosystems is probably the one that has caught major attention (i.e. GESAMP, 1993; Neff et al., 2000; Jernelov, 2010). Aquatic pollution by petroleum is usually caused by spills from tankers or industrial discharges; it is critical in rivers and coastal waters. Although the most visible feature of oil spill is the surface slick or mousses, the water soluble hydrocarbon fraction (WSF) is, in fact, responsible for the toxic effects.

The use of biomarkers in environmental toxicology is becoming increasingly important. The need to detect and assess the impact of pollutants, particularly at low, sublethal concentrations has led to the development of a range of biomarkers measured in a number of different species. Invertebrates have received little attention about signaling potential environmental endocrine disruption compared to vertebrates. This can be largely attributed to the shortage of basic knowledge on their endocrine systems. Nevertheless, in the last few years, knowledge on the control of vitellogenesis in arthropods has been enlarged mainly in insects and crustacean, that have become appropriate models to investigate those mechanisms dealing with the hormonal control at cellular and molecular levels (Billinghurst et al., 2000; Tuberty et al., 2002).

Egg yolk proteins, lipids and carbohydrates in are the major sources of energy and structural compounds for embryo development. In aquatic invertebrates, these compounds are usually associated, forming lipo-glyco-carotenoprotein complexes called lipovitellins (LV) that provide for embryo nutrition and also for larvae metabolic needs from hatching up to the moment they start feeding on external sources (Lee, 1991). Lipovitellins originate from vitellogenins (VTG), which are circulating lipoproteins in vitellogenic females. Recently, several studies have focused on the identification and purification of crustacean LV to establish toxicity tests in crustacean species such as mysids (Tuberty et al., 2002), prawns (Chang et al., 1996; Chen and Kuo, 1998; Kawazoe et al., 2000) and shrimps (Oberdorster et al., 2000).

The freshwater prawn *Macrobrachium borellii* is a decapod crustacean widespread in South America that lives in turbid, tepid waters in the Río de la Plata estuary (Boschi, 1981; Morrone and Lopretto, 1995). It posess one circulating VTG and one egg LV, whose lipid and protein composition have been previously characterized (García et al., 2002, 2004). LV and VTG were found to be immunologically related and with a similar apoprotein composition (García et al., 2008).

It has been well established in several species that VTG synthesis is altered by environmental stressors, therefore it is a widely used biomarker in studies dealing with endocrine disruption induced by xenobiotics (Gagnaire et al., 2009). However, work to evaluate potential use of VTG from invertebrates as a biomarker

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of hydrocarbon pollution has only being reported in a species of the mussel *Mytilus*, and there is no report on the use of VTG from arthropods in this regard.

The aim of the present study was to evaluate the potential use of VTG as biomarker of exposure to hydrocarbon of the prawn *M. borellii*. First, we described the hemolymph VTG and egg LV levels during ovarian development. Then, the effect of sublethal concentrations of WSF on VTG and LV synthesis at different ovaric maturation stages was analysed and the most sensitive vitellogenic stage identified.

2. Materials and methods

2.1. Lipoprotein isolation

Lipoproteins of vitellus were isolated by density gradient ultracentrifugation as previously described (Heras and Pollero, 1990). In short, samples were overlayered on NaBr solution (density 1.26 g/ml) containing 0.01% sodium azide, and centrifuged at 178,000 × *g* at 10 °C for 24 h in a Beckman L8 70 M centrifuge, using a SW 60 Ti rotor. Saline solution of the same density as that of samples was centrifuged in parallel to determine relative densities and to check the correct gradient formation. The total volume of the tubes was fractionated from top to bottom into 0.2 ml aliquots, and the protein content of each fraction was monitored spectrophotometrically at 280 nm. The gradient zone containing lipoproteins was separated as a whole fraction. All the samples were frozen at −70 °C until analysis.

2.2. Sampling

Pre-ovogenic female adult specimens of *M. borellii* of similar size and weight (40 ± 5 mm and 1 ± 0.2 g) were collected in August in a water course close to the Río de la Plata river, Argentina. Females were taken to the laboratory and kept in dechlorinated tap water at 20 ± 5 °C, under a 14:10 L:D photoperiod with an formulated diet (Collins and Petriella, 1999).

Samples of females were taken along vitellogenesis on a 7-day basis. Tissue samples (ovary, hepatopancreas) were dissected out and homogenized in a Potter-type homogenizer (Thomas Scientific, Swedesboro, NJ) with phosphate buffer 50 mM, pH 7.4 containing 0.8 μM aprotinin (Trasylol, Mobay Chemical, New York, USA). Hemolymph was obtained by puncturing the cephalic sinus using a syringe containing 0.1 N sodium citrate as anticoagulant.

Female specimens at different stages of ovary maturation were collected weekly from a water course close to the Río de la Plata river, Argentina from September to November to draw correlations between gonadosomatic index (GSI) and the amount of VTG/LV.

GSI was calculated as the gonad weight (g) × 100/body weight (g). Total protein concentration was measured colorimetrically by the method of Lowry (Lowry et al., 1951).

2.3. Preparation of the WSF of crude oil

Punta Loyola light crude oil from Santa Cruz, Argentina, was stored at 4 °C and used to prepare the water-soluble fraction. The procedure was a modification of that applied by Heras et al. (1992). Crude oil and freshwater in a ratio of 1:100 (v/v) were stirred for 24 h in a 10 L stainless steel mixing vessel equipped with a mechanical stirrer, a bottom drain, and allowed to settle for an additional 48 h. The WSF kept in a cold room at 4 °C was collected daily using the bottom drain. During experiments, fresh WSF batches were prepared every 2 days using several 10 L vessels to compensate the volatilization of lighter hydrocarbons (Heras et al., 1995).

2.4. WSF exposure

The effect of WSF on vitellogenin synthesis was evaluated using adult female prawns at different degrees of ovarian maturation. They were exposed to sublethal concentrations of WSF (0.6 ppm, selected from the results of a previous study (Lavarías et al., 2004), for 7 days without feeding, and the WSF was replaced daily. Another group of females at a similar ovaric maturation stage was kept under the same conditions without the addition of WSF. After exposure prawns were processed separately, dried on adsorbent tissue, weighed on a semi-analytical scale with 0.01 mg accuracy, and dissected and ovaries were kept at −70 °C until used.

2.5. Preparation of anti-LVantibody

Antibodies directed against purified LV were prepared in rabbits as described before (García et al., 2008).

2.6. Enzyme-linked immunosorbent assay (ELISA)

The procedure was based on the assay of Engwall and Perlmann (1972). The standard curve was prepared using purified LV. Nunc-Immunoplate Polisorp microtiter plates were loaded with 50 μL/well of the LV standard (0–120 ng) dissolved in a buffer containing 15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6 (coating buffer). Samples of hemolymph, ovary, hepatopancreas and eggs were diluted with the coating buffer. Aliquots of 50 μL were pipeted into the wells and incubated at 37 °C for 90 min. The antigen solutions were then shaken out, and each well was filled with 300 μL of PBS, pH 7.4, containing 3% (w/v) non-fat dry milk. The plates were incubated at room temperature for 2 h and subsequently washed three times with 0.05% (v/v) Tween in PBS. The anti-LV rabbit serum diluted in PBS-Tween (1:1000) containing 3% non fat dry milk was poured into each well, and plates were incubated overnight at 4 °C and washed three times as above indicated.

Goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad Laboratories) diluted (1:1000) in PBS-0.05% Tween-3% non fat dry milk was added to each well (50 μL) and incubated at 37 °C for 2 h. After four washes as before, 50 μL aliquots of substrate solution, ABTS, and H_2O_2 (Bio-Rad Laboratories) were added to each well, and the plates were incubated at room temperature for 15 min. After color development, the reaction was stopped by the addition of 2% oxalic acid (50 μL) and the absorbance was read at 405 nm on a Beckman Coulter, Inc. Instruments. One percent of non fat dry milk in PBS was used in all the assays as a negative control and blank. All samples were analyzed in triplicate and the values were averaged.

2.7. Statistical analyses

The samples were analyzed in triplicate and standard deviations calculated were apropiate. At least three separate experiments were performed for each study unless otherwise stated. Data were analyzed by Studenťs *t*-test using Instat V 2.0. Results were considered significant a $p < 0.05$.

3. Results

3.1. Dose–response titration of hemolymph and tissues from M. borellii

The anti-LV antibody was used to develop an ELISA for detecting LV and the immunologically related VTG. The standard curve evidenced a linear range of 5–120 ng/well for the purified LV whereas linearity was 50–794 ng/well in hemolymph and 0.3–7.9 μg/well

in ovary homogenates. No cross-reaction with hepatopancreas' proteins within the range 0.1–7.9 μg/well was observed. Ovary and hemolymph titration curves displayed a linear regression (r^2 = 0.71) while no LV-reacting proteins were detected by ELISA in the hepatopancreas of ovogenic females (Fig. 1).

3.2. Variation of IGS, LV and VTG levels throughout ovary development

Vitellogenesis (followed by ovary maturation) was measured by IGS. Fig. 2A shows small increments of IGS (1.7 and 3.1) during the first 35 days, reaching the maximum on day 42 with a value of 8.14. The index then dropped to almost 0 during spawning, which begins around day 55 of vitellogenesis.

The amount of vitellogenin was correlated with the IGS increment and maximums around 220 μg/ml were recorded on day 40 ± 5 (Fig. 2B). Likewise, the amount of lipovitellin in ovary steadily increased reaching a maximum of 450 μg/OV at 35 days (Fig. 2C).

The relationship between the levels of hemolymph (VTG) or ovarian (LV) lipoprotein levels and vitellogenesis stage was determined in females collected from a natural environment. The relationship showed a sigmoid behavior with a maximum around 200 μg/ml VTG for GSI greater than 6 (r^2 = 0.95) (Fig. 3A). On the contrary, LV ovarian levels showed a linear correlation with GSI between 150–790 μg/OV (r^2 = 0.95) (Fig. 3B). In these females the concentration of hemolymph VTG increased earlier than that of ovarian LV.

3.3. Effect of exposure to WSF on VTG and LV along ovarian development

After the description of LV and VTG levels along ovarian development a 7-day exposure of females to WSF at different stages of ovary development (expressed as GSI) was carried out. Although at all developing stages the exposed females displayed a higher LV values, a significant increment of LV concentration varying between 14% and 40% was found for females with gonadomatic indexes higher that 7.4 (Fig. 4). On the other hand, the effect of WSF on circulating VTG along vitellogenesis showed that females with IGS higher than 7.4 evidenced a highly significant increment in VTG concentration, which varied between 52% and 180% as compared with control females (Fig. 5).

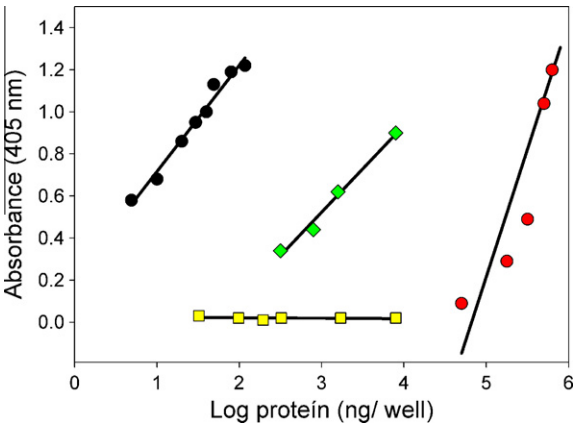

Fig. 1. Dose–response titration of hemolymph and tissues in *M. borellii* using ELISA. Wells were coated with various amounts of proteins from either ovarian extract (□), hepatopancreas extract (◇) or hemolymph of vitellogenic females (●). A standard curve using purified LV (△) was used for quantitation. Purified LV ranged between 5–120 ng/well.

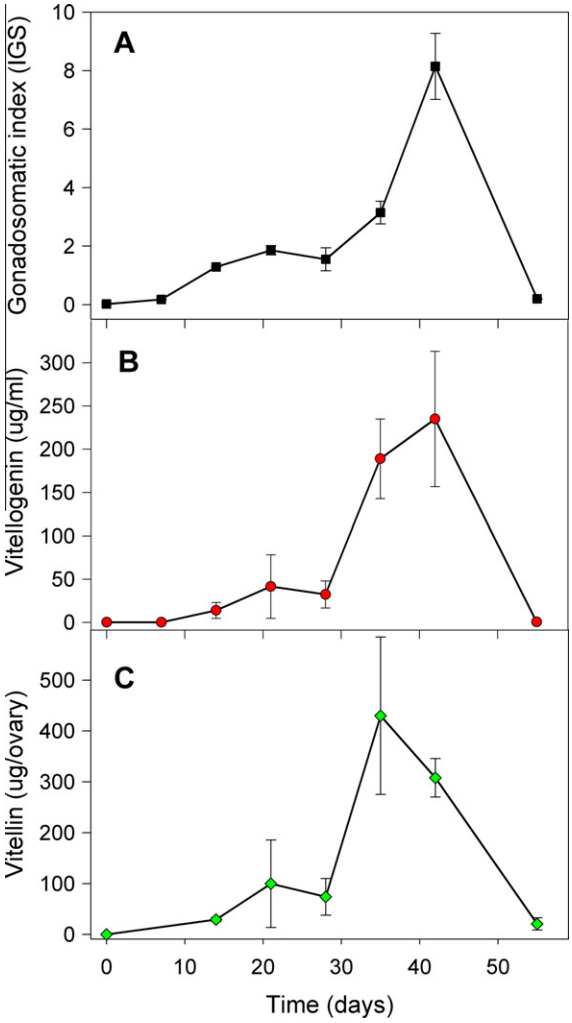

Fig. 2. Changes in the gonadosomatic index (IGS) (A), vitellogenin levels (B) and lipovitellin levels (C) in females of *M. borellii* along development (days). Values represent the mean ± 1 SD (n = 6).

4. Discussion

4.1. Changes in vitellogenin and lipovitellin levels with reproductive status

The immunological relationship between hemolymph VTG and ovarian LV has been previously demonstrated in *M. borellii* as well as in other crustaceans as well as its incorporation into the ovary (Vazquez-Boucard et al., 2002; Auttarat et al., 2006). Previous reports have shown that circulating VTG, ovarian LV and embryo LVe from *M. borellii* are immunologically and electrophoretically similar lipoproteins, containing two major apolipoprotein subunits of 112 and 94 kDa (García et al., 2008).

The affinity of anti-LV antibody for *M. borellii* lipoproteins was confirmed by ELISA assays of crude extract samples containing the unpurified antigen and by the use of purified antigen. The method showed a linear dose–response within a range of 4–120 ng which is narrower than that reported for *Litopenaeus merguiensis* (30–300 ng) (Auttarat et al., 2006) or *Ccllinectes sapidus* (62–1500 ng) (Lee and Watson, 1994), but still adequate for the analysis of the response to pollutants.

Vitellogenin concentration in *M. borellii* females was found to be on average 250 μg/ml, which lies within the very variable concentration reported for decapod crustaceans. In fact VTG values in

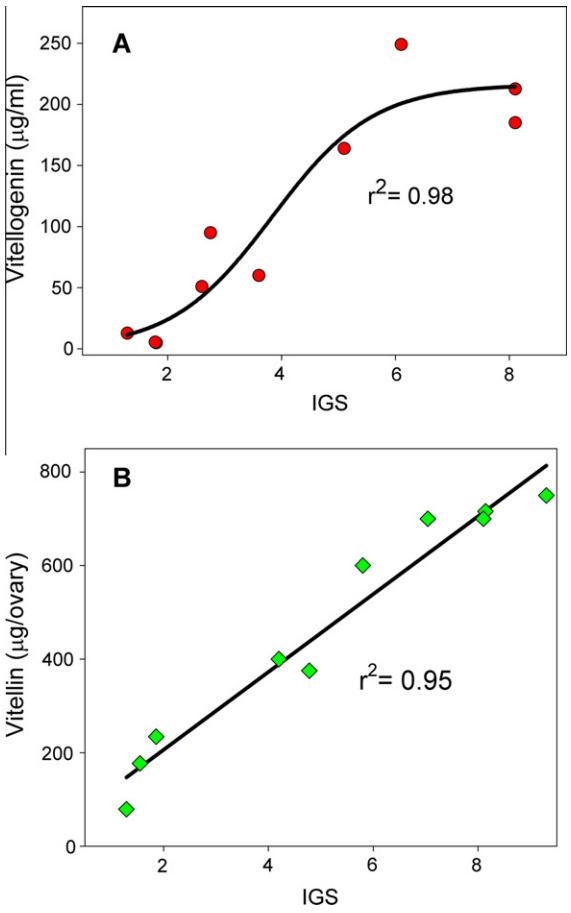

Fig. 3. Relationship between GSI and vitellogenin (A) or lipovitellin (B) levels on females at different vitellogenic stages in the freshwater prawn *M. borellii* ($n = 10$).

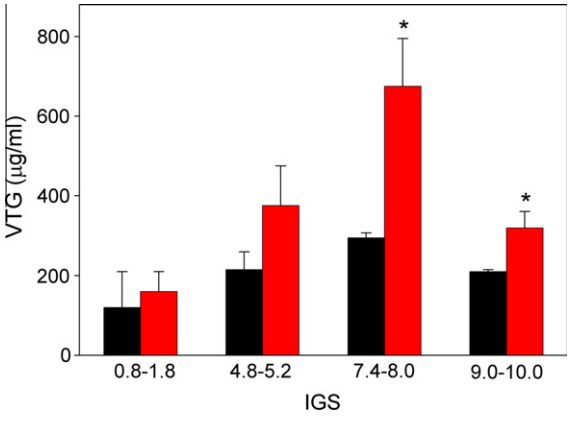

Fig. 4. Effect of a 7 day WSF exposure of females at different developmental stage on lipovitellin concentration in ovary. ■ Control prawn; ■ WSF-exposed prawn. Bars represent the mean ± 1 SD, $n = 6$ (*) $p < 0.005$.

crustaceans are as low as 10-fold lower concentration than that in *M borellii*, e.g. 28.8 µg/ml in *Cancer antennarius* (Spaziani, 1988) to 592–700 µg/ml in *Penaeus semisulcatus* and *Homarus americanus* (Shafir et al., 1992; Tsukimura et al., 2000). The highest recorded values are 7000–8000 µg/ml in *Penaeus monodon* and *Penaeus vannamei* (Quinitio and Millamena, 1992; Yano and Chizei, 1987). This wide variation in the amount of VTG in crustaceans may be related with the size of the organisms and probably with other factors such as the varied sites of VTG synthesis in this taxa.

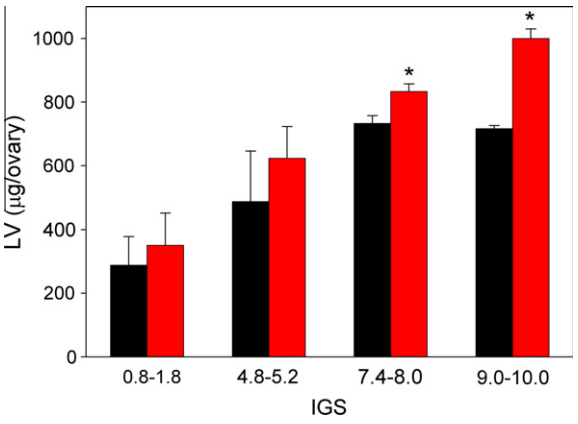

Fig. 5. Effect of a 7 day WSF exposure of females at different developmental stage on vitellogenin concentration. ■ Control prawn; ■ WSF-exposed prawn. Bars represent the mean ± 1 SD, $n = 6$ (*) $p < 0.005$.

The levels of hemolymph VTG in *M. borellii* are in agreement with the amount of lipoprotein taken up by the ovary, and eventually with the LV incorporated into the eggs as previously reported (García et al., 2008).

The present study showed that vitellogenin levels increased in a sigmoidal-shape curve together with ovary development up to an IGS of 6.0 (approx. 38 days), then hemolymph values remained constant until oviposition. This VTG behavior was found in other crustaceans as *L. merguiensis, Sicyonia ingentis* and *Marsupenaeus japonicus* (Auttarat et al., 2006; Tsukimura et al., 2000; Okumura et al., 2006). Circulating VTG is eventually transferred into the ovary where it accumulates as LV; and it was observed that this increment is continuous throughout the ovary development. The same pattern was observed in *L. merguiensis* (Auttarat et al., 2006) as well as in experiments of eyestalk ablation in *M. japonicus* (Okumura et al., 2006).

This behavior may indicate that VTG concentration reaches an early maximum or that its synthesis is faster than LV accumulation. A similar result was reported by Lee et al., when they studied the relationships among IGS, VTG and LV in *Macrobrachium rosenbergii*. In this species, VTG concentration decreased in females with a very mature ovary (IGS > 8) (Lee et al., 1997). This similar behavior between *M. borellii* and *M. rosenbergii* was expected, considering that lipoproteins, LV in *M. borellii* and VTG in *M. rosenbergii* evidenced high sequence homology as assessed by MALDI-TOF analysis (García et al., 2006).

4.2. Effect of hydrocarbon exposure on vitellogenin and lipovitellin at different reproductive status

The effect of the hydrosoluble fraction of light crude oil (WSF) was evaluated at three life stages of *M. borellii*. Previous reports have shown that *M. borellii* early life stages are not very sensitive to WSF toxicity while adult specimens display a quick uptake and release of the contaminating hydrocarbons from the media and are therefore more suitable as markers of contamination (Lavarías et al., 2004). Also, it was assessed that catabolic enzymes can be used as biomarkers of early pollution as WSF increases the energy requirements in *M. borellii* (Lavarías et al., 2006).

In recent years studies dealing with contaminants in aqueous environments have been increased and included in programs of prevention of environmental pollution, and several chemicals have been recognized as endocrine disruptors in invertebrates (Billinghurst et al., 2000). However, little work have been done on the effect of crude oil hydrocarbons on invertebrate VTG levels, being

restricted to mussel species (Ortiz-Zarragoitia and Cajaraville, 2006; Aarab et al., 2004).

The present study shows for the first time that the exposure of adult crustacean to sublethal concentrations of WSF significantly increase VTG and LV in hemolymph and ovary, This may be produced by polycyclic aromatic hydrocarbons which could be considered estrogenic chemicals, as organochloride pesticides, polychlorinated biphenyls and surfactans are (Sumpter, 2005). We have previously determined that the levels of polyclyclic aromatic hydrocarbons of the WSF utilized in our experiments, is approximately 7 μg/L which would be enough to elicit estrogenic-like effects (Lavarías et al., 2004).

Some studies have dealt with the induction of protein-like VTG by the presence of freshwater hydrocarbon oil toxicants on fish. For instance, in rainbow trout (*Oncorhynchus mykiss*) (Knudsen et al., 1997) and tilapia *Oreochromis niloticus* exposed to aromatic hydrocarbons that can be found in rivers (Rodas-Ortiz et al., 2008).

Regarding the use of VTG as a biomarker in invertebrates, studies on the effect of hydrocarbons on protein-like-vitellogenin are restricted to the bivalve mollusk *Mytilus edulis* exposed to crude oil (Ortiz-Zarragoitia and Cajaraville, 2006; Aarab et al., 2004) where the amount of vitellogenin was determined using alkali-labile phosphate, an indirect marker of vitellin. Though this assay is considered as an alternative assessment of ELISA, this method should be applied with caution as alkali-labile phosphorus is a minor part of the total phosphorus pool in crustacean vitellogenin (Volz et al., 2002), and the presence of other phosphorus sources can substantially affect the results (Stanton, 1968).

In conclusion, the study showed that the behavior of reproduction-related lipoproteins is closely related to ovarian development in *M. borellii*. Besides, it also provides the first evidence of the usefulness of measuring the levels of either circulating VTG or ovarian LV as a biomarker of exposure to hydrocarbon pollution in crustaceans and identified the most sensitive reproductive stage. As a whole, we can conclude that changes in VTG-like proteins could be used as potential indicator of pollutant effects on crustacean reproduction in freshwater environments.

5. Disclosure statement

All authors have participated in the research and article preparation.

All authors have approved the final article.

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