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Development of biomarkers of stress related to endocrine disruption in gastropods: Alkali-labile phosphates, protein-bound lipids and vitellogenin-like proteins

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ABSTRACT

The occurrence in aquatic environments of compounds capable of acting as endocrine disruptors (ED) is of concern for wildlife. Elevated levels of the egg-yolk precursor protein vitellogenin (Vg) are widely accepted as a biomarker for xenoestrogen exposure in aquatic organisms. The purpose of this study was to develop a method for tracking changes in levels of Vg-like proteins in gastropods. Vg-like and egg-yolk proteins were analyzed in three freshwater gastropods having different modes of reproduction: *Potamopyrgus antipodarum* (asexual reproduction), *Valvata piscinalis* (hermaphroditism) and *Lithoglyphus naticoides* (sexual reproduction). Vitellogenin-like protein levels were examined by the alkali-labile phosphate (ALP) technique, a novel protein-bound lipid (PBL) assay, and by gradient gel electrophoresis with silver staining. The first phase of the study was dedicated to the development and optimization of an analytical method for detecting Vg-like proteins in these three gastropod species. In the second phase, the snails *P. antipodarum* and *V. piscinalis* were exposed to bisphenol A (BPA), octylphenol (OP) and tributyltin (TBT) for 14 and 28 days. Vg-like proteins were resolved in one major band at 250–300 kDa for *L. naticoides* and *V. piscinalis* and in two bands at 100 and 30 kDa for *P. antipodarum*. After 14 days of exposure, all techniques showed an increase in Vg-like protein levels at 100 µg/L BPA and at 1 µg/L OP in *P. antipodarum*. A decrease in these proteins was observed with high concentrations of OP (100 µg/L) and TBT (≥5 ng/L). In *V. piscinalis*, a decrease in Vg-like proteins was shown after 14 days of exposure for OP ≥10 µg/L and TBT ≥5 ng/L; however, at 28 days, gel electrophoresis revealed an increase in these proteins. Histological observations showed significant necrosis in ovotestes of *V. piscinalis* with the three endocrine-disrupting compounds, while tissue modifications were not detected for *P. antipodarum*. A method for measuring Vg-like and egg-yolk proteins is proposed to track changes in both estrogenic and androgenic endocrine disruptors in gastropods. The potential use of these species as bioindicators of endocrine disruption in freshwater environments in terms of their reproduction mode is discussed.

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1. Introduction

A number of xenobiotics in the environment are able to cause adverse effects in animals and humans by interfering with endocrine function. Endocrine disruptors (EDs) can also lead to a lower sperm quality and increase frequency of cancers and

genital abnormalities (Kavlock et al., 1996). EDs represent a structurally diverse group of compounds which include natural and synthetic hormones (17β-estradiol [E2] and ethynylestradiol [EE2]) and organic compounds originating from industry and agriculture, including polychlorinated biphenyls and organochlorine pesticides as well as miscellaneous chemicals such as plasticizers and surfactants (alkylphenols, phthalates, phenolic compounds, etc.). The chemical bisphenol A (BPA) has one of the highest production rates in the world. It is mainly used by the plastic industry in the production of polycarbonate and epoxy resins. Concentrations of BPA in surface waters have generally been detected in the range of 0.001–1 µg/L, although levels up to 8 µg/L have been found downstream of industrial sewage discharge points (Larsen

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et al., 2006). Alkylphenol ethoxylates of nonylphenols (NPs) and of octylphenols (OPs) are used in various industrial processes (e.g. wool washing), but have been banned in household detergents in the European Union. OP concentrations in effluents discharged by sewage treatment plants can reach 0.4–1.8 µg/L (Langston et al., 2005). BPA and OP were shown to induce superfeminization in the gastropod *Marisa cornuarietis* at environmentally relevant exposure concentrations. The effects of superfeminization include formation of additional female organs, enlarged accessory sex glands, gross malformations of the pallial oviduct, and stimulation of egg and egg-mass production resulting in increased female mortality (Oehlmann et al., 2000). Tributyltin (TBT) is one of the most powerful antifouling biocides and has been widely used on ships in both saltwater and freshwater environments since the mid-1960s (Alzieu, 2000). The use of TBT as an antifouling agent was banned in paint used on small boats and fishing nets in most countries due to its toxicity and potent endocrine-disrupting effects on aquatic biota. The most striking effect of TBT is the development of male organs in gastropod females, a phenomenon known as *imposex* (Bettin et al., 1996). This syndrome can lead to sterilization and death and has been shown in more than 150 species of gastropods (Matthiessen and Gibbs, 1998).

One of the most documented effects of xenoestrogens is the induction of the egg-yolk protein precursor vitellogenin (Vg), which has been extensively used as a biomarker of endocrine disruption both in laboratory and field studies on fish (Porte et al., 2006), and also more recently in bivalves (Matozzo et al., 2008). Vg is the major precursor of the egg-yolk proteins vitellins (Vn), which provide energy to support embryonic development in oviparous organisms. They are large, high-molecular-weight (300–600 kDa, depending on the species) proteins, rich in glucose (glycosylated), lipids, phosphates and containing Ca and Zn as ligands. In mature female vertebrates, they are normally synthesized in the liver (or equivalent tissues) in response to endogenous estrogens such as 17β-estradiol and transported via the blood to be stocked in the oocytes (Nagler et al., 1987). Vgs have also been shown to be regulated by natural and xenoestrogens in bivalves: increased levels of these vitellin proteins have been reported in the marine clam, *Mya arenaria* (Blaise et al., 1999), the freshwater mussel, *Eliptio complanata* (Gagné et al., 2001), and the Pacific oyster, *Crassostrea gigas* (Li et al., 1998), exposed to estradiol, municipal effluent, and nonylphenol, respectively. Methods employed to assay Vg levels have traditionally relied on immunoassays for fish (Jobling et al., 2004). However, these techniques are not as well developed in invertebrates, including bivalves and snails: there are few specific antibodies developed against bivalve Vg, and these antibodies usually show low cross-reactivity across species (Li et al., 1998; Blaise et al., 1999). Therefore, alternative indirect assays such as the organic alkali-labile phosphate (ALP) assay and gel electrophoresis analysis have been developed (Matozzo et al., 2008). The ALP method, based on the determination of labile phosphates released by Vg after hydrolysis in alkali, was shown to be significantly correlated with the other direct assays on fish (Nagler et al., 1987) and bivalves (Gagné et al., 2004). The release of inorganic phosphates can therefore be considered as biomarker of response to estrogens, even though it cannot provide quantitative measures of egg-yolk protein concentrations (Gagné et al., 2002). Recently, studies showed that the lipid content of Vg could also be used as another indirect means to measure egg-yolk proteins (Gagné et al., 2002).

Most data on the biological effects and mechanisms of action of endocrine disruptors on marine organisms are taken from studies on vertebrates. Although invertebrates constitute roughly 95% of all animal species and are key components of most ecosystems, few studies have examined the effects of estrogenic EDs on invertebrates (Brennan et al., 2006). Nevertheless, imposex in gastropods

is considered the most dramatic example of endocrine disruption in wildlife. The lack of scientific data on invertebrates is partly due to the limited knowledge on invertebrate endocrinology and the lack of bioanalytical methods. Although vertebrate-like sex steroid hormones have been discovered in many invertebrate groups, their function, in most cases, remain equivocal (Matthiessen et al., 1999; Lafont and Mathieu, 2007). Thus, there are few direct biomarkers of endocrine disruption in such species. Steroid levels were sometimes shown to be altered in bivalves and snails exposed to xenobiotics (Gauthier-Clerc et al., 2002; Siah et al., 2003; Janer et al., 2006), but it remains unclear whether this was due to specific action on the endocrine system or the result of indirect toxic action.

Prosobranch molluscs are ubiquitously distributed, the high number of species being highly significant to the biodiversity and ecological relevance of freshwater ecosystems (Mouthon and Charvet, 1999). Ironically, however, prosobranchs have played a minor role in ecotoxicological research, although they have been successfully used to obtain information on the quality of freshwater ecosystems (Oehlmann and Schulte-Oehlmann, 2003). Prosobranchs should be considered for research on endocrine disruptors because of their differing modes of reproduction, such as hermaphroditism and parthenogenesis (Oehlmann et al., 2007), in addition to the sexually dimorphic species. In this study, the test species were the freshwater mudsnails *Potamopyrgus antipodarum* (Hydrobiidae), *Valvata piscinalis* (Valvatidae) and *Lithoglyphus naticoides* (Hydrobiidae). *P. antipodarum* has diploid parthenogenetic reproduction (Robson, 1923). In New Zealand, where these snails originate from, sexual reproduction can appear when parasites threaten the balance of the population, while the European population is almost exclusively female (Ponder, 1988; Jokela et al., 1997). The species is ovoviviparous. The spawning occurs all year long with two maxima in December and April. The number of embryos per spawn is variable (20–60 embryos in the brood pouch). The life cycle of *V. piscinalis* is annual. Reproduction starts in June and occurs until the end of summer (Cleland, 1954). Animals spawn sticky and rigid egg capsules (1 mm diameter), containing 15 eggs, spawned on vegetation. Juveniles come out the capsule 12–30 days after spawning. Reproduction is hermaphroditic; animals are first protandrous, then male and female simultaneously (Cleland, 1954). The life cycle of the *L. naticoides* population in France starts with a spawning from March to April; adults disappear in July. The new cohort appears in May and June and reaches its adult size in October. *L. naticoides* only gives birth to one cohort per year and its lifespan varies from 13 to 15 months. The peak density occurs in June. The sexes are separated (dioecious). *L. naticoides* generally spawn eggs (one individual per egg) on the shells of their congeners (Mouthon, 2007).

The aim of this study was to characterize the effects of endocrine disruptors on Vg-like protein levels in freshwater gastropod molluscs. Indirect (alkali-labile phosphate and protein-bound lipids [PBL]) and direct (gel electrophoresis) methods were used in the first phase to detect Vg-like proteins in control specimens of these species. In phase two, *P. antipodarum* and *V. piscinalis* were challenged to BPA, OP and TBT for a four-week period. Reproduction (neonates of *P. antipodarum*, egg capsules of *V. piscinalis* and histological observations) was tracked and animals were sampled in order to measure the effects of endocrine disruptors on Vg-like proteins.

2. Materials and methods

2.1. Chemicals

Bisphenol A, octylphenol, and tributyltin chloride (TBT) were purchased from Sigma–Aldrich (St. Quentin-Fallavier, France).

2.2. Snail collection and maintenance

As part of the developmental experiment, *P. antipodarum* and *V. piscinalis* were obtained from laboratory cultures (CEMAGREF, Lyon, France). Adult *L. naticoides* were sampled in the Saone River in France (Mouthon, 2007). In preparation for the exposure experiments, 800 gastropods of each species were collected in the field (Rhône River for *P. antipodarum* and Saone River for *V. piscinalis*) and allowed to acclimate for two weeks. Animals were reared under the usual conditions in aerated glass aquaria (17–20 L) at a temperature of 22 ± 1 °C and under a 16/8 h artificial light/dark photoperiod. Animals were fed using ground commercial fish food (Tetramin®). Adult snails of similar size ($3.5 \text{ mm} \pm 0.2$ for *P. antipodarum* and *V. piscinalis*) were used for the exposure experiments; juvenile snails of similar size ($2 \text{ mm} \pm 0.2$ for *P. antipodarum* and *V. piscinalis*) were used for the developmental experiments. Adult *L. naticoides* were $4.5 \pm 0.5 \text{ mm}$.

2.3. Method development for egg-yolk analysis

2.3.1. Snail handling and tissue preparation

Ten adults of *P. antipodarum* were dissected under a binocular microscope and the embryos in the brood pouch were sampled (Duft et al., 2003b). Briefly, the shell was cracked using a holding vice, the brood pouch was carefully collected and the embryos were removed using a Pasteur pipette. The embryos from 10 adults were pooled and three groups of pooled (i.e. 10) adults were prepared. Three pools of 30 juveniles were also prepared. Juveniles were not dissected. Adults, juveniles and embryos were weighed and stored at -80 °C until biochemical analyses for vitellogenin-like proteins. For *V. piscinalis*, three pools of 10 adults and of 30 juveniles were also prepared. As above, adults were dissected (removal of shell) while juveniles were not. Egg capsules were also collected and stored at -80 °C until the analyses. Finally for *L. naticoides*, the adults were sexed under a binocular microscope in live animals. Three pools of five males and five females were prepared. Animals were dissected under a binocular microscope as described above and eggs were also collected. Males, females and eggs were pooled, weighed and stored at -80 °C.

2.4. Exposure experiments

2.4.1. Exposure to test substances

No *L. naticoides* were available for the contamination experiment. *P. antipodarum* and *V. piscinalis* were concomitantly exposed to three concentrations of each pollutant (i.e. 1, 10, 100 µg/L for OP and BPA, and 5, 50, 500 ng/L for TBT (nine treatments groups)). The solvent vehicle was 0.05% acetone and it was kept constant for each exposure concentration.

Thirty snails of each species were placed in beakers containing 0.6 L of clean, filtered (1 µm pore-filter membrane) river water obtained from the Bourbe River (Isère, France), using three replicates for each condition (i.e. $n=3$ beakers per treatment group). The beakers were maintained at 18 ± 1 °C with a 16/8 h light/dark photoperiod using a water bath. The test water in the beakers was continuously renewed (2.5 renewals a day): for each concentration, a continuous pumping of clean water and stock pollutant solutions were premixed before being added to the beakers. A river water control and a solvent control were also included (three replicates each). The exposure period lasted 28 days. Organisms were fed daily with 0.35 mg/individual Tetramin® per working day. During the exposure period the number of dead snails was recorded and they were removed on a weekly basis. Samples of water from the experiment and from the initial solutions of pollutants were also taken at the beginning of the experiment in order to monitor pollutant concentrations.

2.4.2. Measurements of pollutant concentrations in water samples

2.4.2.1. Organotin analysis. Freshwater samples were stored frozen at -20 °C in the dark to await laboratory analysis; the extraction protocol is detailed in Dévier et al. (2003). Daily, a solution of 50 µL of 100 ng Sn mL⁻¹ tripropyltin chloride (TPrTCl) was added to a 100-mL water sample (except 1 L for the 5 ng/L experiment) and used as the internal standard. Ethylation of organotin compounds was performed in a 250-mL narrow-necked (1 cm i.d.) flask (except 1 L for the 5 ng/L experiment) using a magnetic stirring rod. The pH was adjusted to 5 with 5 mL of 1 M sodium acetate buffer. Then, 100 µL of a 2% NaBEt₄ solution (Strem Chemicals, Bischheim, France), previously purified by two successive extractions with isoctane to obtain lower blank values, was added and the mixture was stirred for 10 s. The reaction was allowed to develop for 10 min in the dark before extraction of the ethylated derivatives by means of 500 µL of isoctane with vigorous magnetic stirring for 15 min. Milli-Q water was added as necessary to bring the level up to 3 cm in the narrow neck. After decantation, the 0.5 mL supernatant plus the 1 mL underlying aqueous phase were transferred into a 2-mL Eppendorf tube and centrifuged for 5 min at 10,000 rpm at 4 °C. Aliquots of 200 µL of the isoctane extracts were transferred into autosampler vials with the addition of 50 µL of a daily prepared 100 ng Sn mL⁻¹ tetrabutyltin (TeBT) solution used as the internal standard for recovery determinations. The final extracts were analyzed by gas chromatography/mass spectrometry (GC/MS) within 24 h. Analyses were performed on an HP 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) fitted with a HP5-MS capillary column (5% diphenylsiloxane–95% dimethylsiloxane; 30 m × 0.25 mm × 0.25 µm; BIOS Analytique, L'Union, France). Samples were injected into the gas chromatograph in pulsed splitless mode (30 psi; 1 min) at 280 °C using an HP 6890 series injector. The carrier gas was ultrapure helium (99.99990%; Linde Gas, Bassens, France) set at constant flow mode (1.3 mL/min). The GC temperature program was 60 °C (3 min hold) to 200 °C at a rate of 20 °C/min and then to 280 °C (5 min hold) at a rate of 10 °C/min. The gas chromatograph was coupled to an HP 5973 Mass Selective Detector (MSD; Agilent Technologies, Palo Alto, CA), operated in electron impact (EI) mode at 70 eV under the single ion monitoring mode (SIM) at 1.2 scan/s, using one quantification and three qualifier ions for each compound. The transfer line, source and quadrupole temperatures were 280, 230 and 150 °C, respectively. Quantification was performed by internal calibration relative to tripropyltin. The relative response factors of the different compounds were determined by injecting a standard solution of butyl- and phenyl-tins (individual standard solutions prepared from pure crystals) spiked with the same internal standard solution as that used to spike the samples.

2.4.2.2. BPA and OP analysis. Bisphenol A and octylphenol contents were determined in standard stock solutions used for the exposure experiments. Standard solutions were stored frozen at -20 °C in the dark until the laboratory analyses. Following appropriate dilution, samples were directly analyzed by liquid chromatography/mass spectrometry (LC/MS) (HPLC 1100 series, G1946VL MSD, Agilent Technologies) (Cailleaud et al., 2007). The separation of compounds was performed at 0.15 mL/min on a reverse-phase Kromasil C18 column (150 × 2.1 mm × 3 µm; Interchim, France) with a binary mobile-phase consisting of methanol/5 mM ammonium acetate in Milli-Q water (33/67, v/v) (A) and pure methanol (B). The elution gradient was 60% of B for 2 min, linearly increased to 80% within 5 min, kept isocratic for 18 min, then linearly decreased to 60% within 1 min, and finally kept isocratic for 10 min. The detection was achieved on a triple quadrupole mass spectrometer equipped with an electrospray interface used in positive ionization mode (ESI+). The source and desolvation temperatures were set at 100

and 350 °C, respectively, and the flow rate of the desolvation gas was set at 10 mL/min. Acquisition was accomplished in SIM mode, and BPA and OP were identified by confirming the characteristic pattern showing [NPnEO–Na]⁺ ions.

2.5. Biological and morphological evaluations

The number of neonates of *P. antipodarum* in each beaker was determined weekly (D0, D7, D21 and D28) using a binocular microscope. Ten animals per beaker were randomly collected every two weeks (D0, D14 and D28) for embryo production in the brood pouch, as described earlier. After counting, the animals were pooled, weighed and stored at –80 °C for subsequent analyses, as described below. The number of released capsules and eggs in each capsule of *V. piscinalis* were determined at the end of each week. Ten animals per beaker were randomly collected every two weeks (D0, D14 and D28), dissected and frozen as described above. At the end of the exposure (D28), five *P. antipodarum* and five *V. piscinalis* were randomly taken for histological analyses. Samples were fixed for 48 h in Bouin's solution, rinsed and dehydrated in alcohol. Snails were thereafter included in Paraplast X-tra® (Sigma–Aldrich). Serial cross-sections of 5 µm were then made and coloured using Hemalun–Eosin (Martoja and Martoja, 1967). Blades were read with a Leica DM 2500® microscope. For both the developmental and exposure experiments, all samples stored at –80 °C were sent on dry ice to Environment Canada (Montreal, Canada) for the analyses of vitellogenin-like proteins.

2.6. Biochemical properties of egg-yolk proteins, gender and snail maturity

Egg-yolk proteins were directly analyzed in acetone-fractionated proteins for vitellogenin-like proteins (by gel electrophoresis), or indirectly using measurements of alkali-labile phosphates (Gagné et al., 2005) and protein-bound lipids (both by spectrophotometry). Briefly, tissue homogenates were prepared on ice in 10 mM HEPES–NaOH, pH 7.4, containing 0.2 M KCl and 1 µg mL^{–1} aprotinin (protease inhibitor at 6000 KIU/mg; Sigma Chemical Company) using a Teflon pestle tissue grinder. The homogenates were centrifuged at 1000 × g for 10 min at 4 °C and the supernatant was carefully collected from the pellet and stored at –80 °C. Protein concentrations were determined in the supernatant by Bradford's method (1976) using serum–bovine–albumin for calibration. High-molecular-weight proteins in the supernatant were precipitated by adding 35% acetone and the protein pellet obtained by centrifuging at 10,000 × g for 5 min at 2 °C. The pellet was resuspended in 100 µL of 10 mM Tris–acetate, pH 8.0, containing 1 mM EDTA and divided between two tubes: one tube for gel electrophoresis and the other for alkali treatment. The alkali treatment consisted of the addition of one volume of 2 M NaOH heated at 60 °C for 30 min. The phosphates and lipids released were determined by colorimetry as described elsewhere (Stanton, 1968; Frings et al., 1972). Calibration was achieved with inorganic phosphate (KH₂PO₄) and Triton X-100 for ALP and protein-bound lipids, respectively. The data were expressed as µg of alkali-labile phosphates or lipids/mg proteins. The proteins were also determined by denaturing polyacrylamide gel electrophoresis using precast high-resolution mini-gels with an acrylamide concentration gradient of 4–12% (Invitrogen, USA). The electrode buffer consisted of 50 mM MOPS buffer, pH 7.2, containing 1 mM EDTA and 0.2% SDS. Samples (i.e. protein pellet resuspension) were then diluted with 50 mM MOPS buffer, pH 7.2, to achieve 10 µg mL^{–1}. An equal volume of loading buffer (Invitrogen, USA) was added to the diluted protein pellet and tubes were heated at 70 °C for 10 min. Then, 10 µL (50 ng) was loaded into the wells and the gels were run for 90–120 min at 90 V. Vitellogenin samples

from the common carp, *Cyprinus carpio*, and the blue mussel, *Mytilus edulis*, were also obtained by the acetone-fractionation procedure and run through the gels. After electrophoresis, the gels were stained with silver using the SilverXpress Kit (Invitrogen, USA). The gels were scanned with a horizontal scanner and band density was determined by the Un-Scan-It software version 6.1 (USA). Molecular-weight markers and aprotinin were also used for calibration of the gels. The data were expressed as ng of protein for each band.

2.7. Statistical analysis

Results were expressed as means ± standard error. Two-way (concentration and time) and one-way (concentration) ANOVAs were performed. Significance was set at $p \leq 0.05$. In the case of rejection of H₀, an *a posteriori* least significant difference (LSD) test was applied. Analyses were performed using STATISTICA software version 7.1. Values were log transformed to achieve normality when necessary.

The relationships among PBL, ALP and female-related high-molecular-weight bands were determined by a hierarchical tree analysis for the method development step owing to the low sample size ($n=8$). The relative distance between each measurement was determined using 1-Pearson's correlation coefficient. For the ED exposure experiments, factorial analyses were performed using the principal component extraction procedure.

3. Results

3.1. Biochemical characterization of snail tissues and egg-yolk proteins

In *L. naticoides*, egg-protein extracts were resolved into a major band at 300 kDa, which was present in females but not in males (Fig. 1). Protein density was significantly higher in both females and eggs compared to males (Fig. 2b). Moreover, a hierarchical analysis of the biomarker data revealed that band density at 300 kDa was closer to the levels of PBL/ALP content than to PBL or ALP (Fig. 3a). This finding indicates that snail egg-yolk proteins (Vg) are essentially lipoproteins rather than phosphoproteins.

In *P. antipodarum*, egg-protein extracts were resolved into a major band at 100 kDa and into a minor band at 30 kDa, both of which were absent in juveniles but readily present in adults (Fig. 1). As shown in Fig. 2a, band density for both bands was significantly higher in eggs and adults as compared to juveniles ($p < 0.01$). Another band appeared at 250 kDa but did not discriminate among eggs, juveniles and adults (Figs. 1 and 2a). A classification analysis showed that bands at 100 and 30 kDa were more closely related to the PBL/ALP ratio than to ALP, PBL or the protein band density at 250 kDa (Fig. 3b).

In *V. piscinalis*, the absence of egg extracts complicated interpretation of the gel electrophoregrams (Fig. 1). A major 250 kDa band was observed but the amount was significantly higher in juveniles than in adults (Fig. 2a). No apparent discriminating bands were observed at 100 kDa. A minor band at 300 kDa was observed but did not discriminate between adults and juveniles (Fig. 1). A classification analysis showed that the 300 kDa band was closer to the biochemical values measured (i.e. PBL, ALP and PBL/ALP ratio) than the 250 kDa band (Fig. 3c).

3.2. Contamination experiment

O₂ and ammonia concentrations were daily measured in all beakers; results showed no major variations of concentrations during the experiment (data not shown).

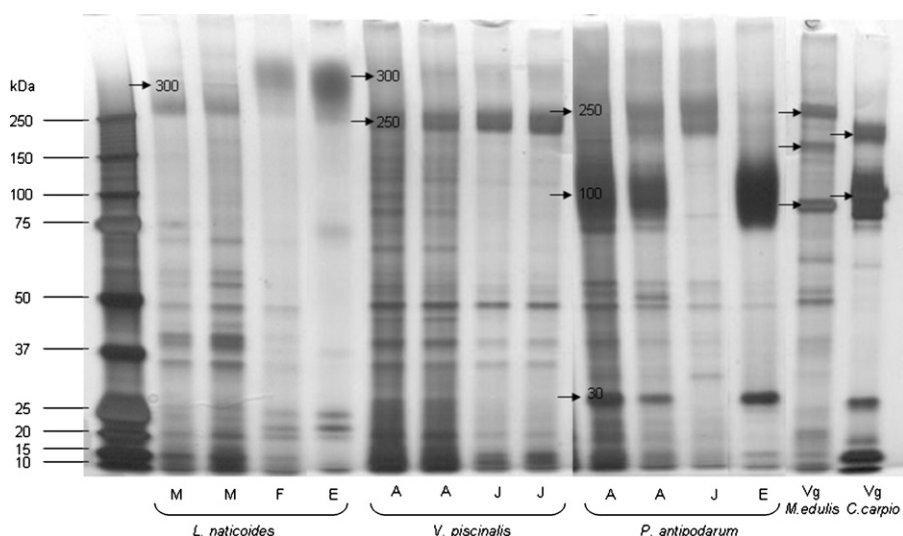


Fig. 1. SDS-PAGE (12%) under reducing conditions of whole-body *L. naticoides*, *P. antipodarum* and *V. piscinalis*. M: males, F: females, E: eggs, A: adults, J: juveniles. The bands considered as vitellogenin-like protein subunits are shown by arrows. Arrows also show the Vg subunits in *M. edulis* and *C. carpio*.

3.2.1. Measurement of real pollutant concentrations in beakers

For TBT, concentrations in beakers were very close to nominal concentrations: the values obtained for nominal concentrations of 5, 50 and 500 ng/L were 5.2, 44.8 and 428.3 ng/L, respectively (variability of 10–15%). For technical reasons, OP and BPA concentrations were only measured in samples taken from the initial solution of 2 mg/mL; measured concentrations of 0.969 ± 0.12 mg/mL for BPA and 0.994 ± 0.45 mg/mL for OP were obtained.

3.2.2. Apical endpoints

The three substances were not lethal to *V. piscinalis*. For the species *P. antipodarum*, significant mortality was observed in organisms exposed to OP but only at the highest concentration of 100 $\mu\text{g/L}$ OP ($p < 0.05$; data not shown) after 28 days of exposure. No effect was observed on the parameters tracked for both species, i.e. the number of neonates and embryos for *P. antipodarum*, and the number of released capsules and eggs in each capsule of *V. piscinalis* (data not shown).

3.2.3. Gonad histology

For *P. antipodarum*, histological sections of gonads from control organisms displayed numerous oocytes at different maturation stages and normal digestive glands. The same structure was observed in animals exposed to all concentrations of the three contaminants (data not shown).

Gonads of control *V. piscinalis* specimens displayed young oocytes, spermatogonia associated with Sertoli cells and clear sperm ducts (Fig. 4a). The same structure was observed in the solvent control specimens (Fig. 4b). Some modifications were shown in ovotestes at the lowest concentrations of BPA and OP (i.e. a thickening of germinal epithelium, necrosed areas and increased numbers of mature oocytes with altered male tissue structures). Animals exposed to 100 $\mu\text{g/L}$ of BPA (Fig. 4c) and 100 $\mu\text{g/L}$ of OP (Fig. 4d) showed severe effects on male tissues, with evident areas of necrosis and the loss of sperm duct structures. This effect was even more evident with OP than with BPA (Fig. 4c and d). For BPA, atretic mature oocytes were also present. TBT (500 ng/L) induced an increase in the number and size of oocytes at different maturation stages in the gonad compared to the controls (Fig. 4e), while no effect on male structure was recorded.

3.2.4. Biochemical endpoints

The levels of PBL and ALP were also determined in both species of snails exposed to BPA, OP and TBT (Fig. 5). The acetone group produced no significant differences in PBL and ALP values.

In *P. antipodarum*, the levels of PBL were significantly increased by BPA, OP and TBT at nominal threshold concentrations ≤ 1 $\mu\text{g/L}$ (geometric mean between the no observed effect concentration [NOEC] and the lowest observed effect concentration [LOEC]). OP had the strongest response, reaching an eightfold increase in PBL (Fig. 5a). The other substances were much less potent in inducing

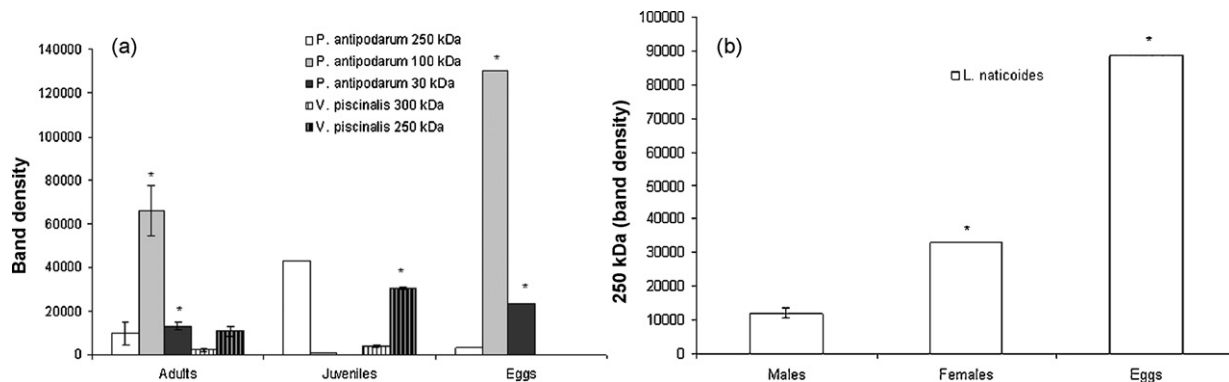


Fig. 2. Band density (number of pixels) in adults, juveniles and eggs of *P. antipodarum* and *V. piscinalis* (a) and in males, females and eggs of *L. naticoides* (b). Values are means of two replicates (two pools of animals, cf. Section 2.3); standard error is presented. $p < 0.05$.

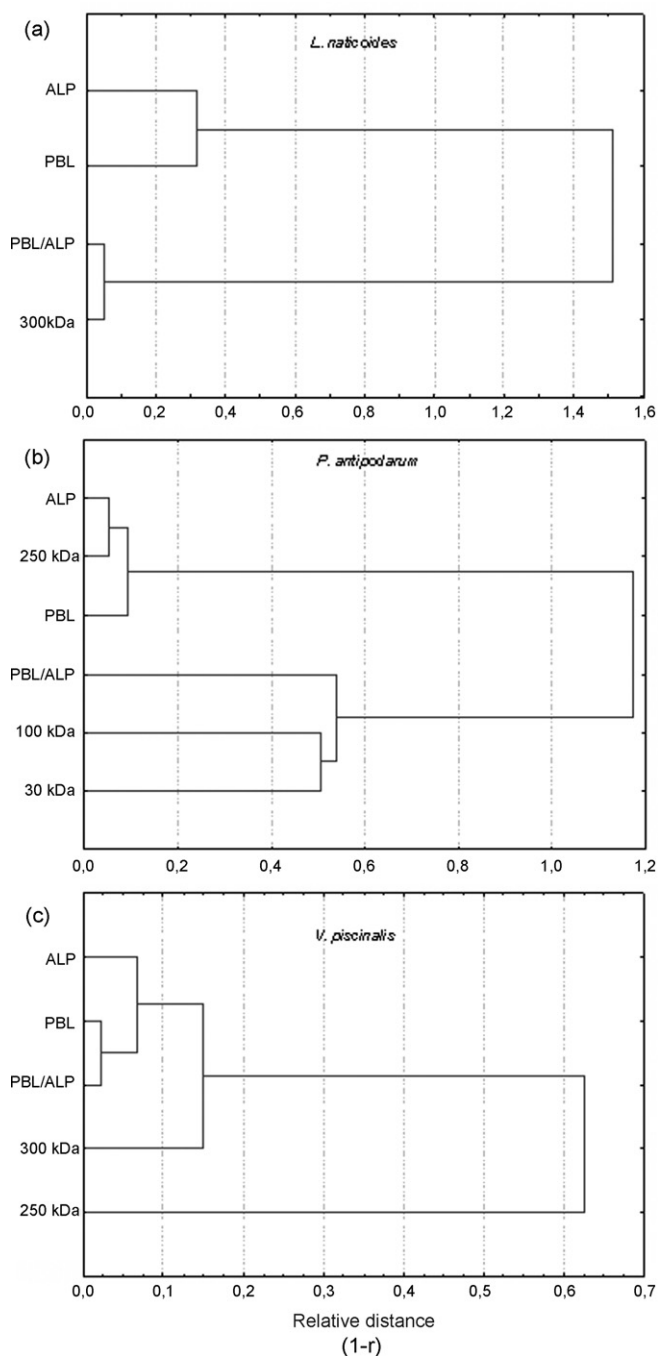


Fig. 3. Hierarchical trees for PBL, ALP, PBL/ALP values and band densities. (a) *L. naticoides*; (b) *P. antipodarum*; (c) *V. piscinalis*. $n=5$ for *L. naticoides* and $n=4$ for *P. antipodarum* and *V. piscinalis*.

PBL, with response factors generally less than twofold. These differences were significant only on day 28 and not day 14. The levels of ALP were also measured in tissues (Fig. 5b). The phosphate levels were significantly higher in BPA and OP-exposed animals at a nominal threshold chemical concentration of $\leq 1 \mu\text{g/L}$. However, the levels returned to control values in exposures with higher nominal chemical concentrations (10 and $100 \mu\text{g/L}$ BPA and OP). TBT produced a more sustained effect in exposures with nominal concentrations three orders of magnitude lower than for the other chemicals, with nominal threshold concentrations $\leq 5 \text{ ng/L}$. These differences were significant only for pooled data drawn from both exposure periods. The PBL data were normalized against the ALP

levels (Fig. 5c) and it was revealed that the oestrogenic compounds (BPA and OP) significantly induced the ratio at D14 where TBT had no effects. No effect was found at D28. A densitometric analysis of protein bands at 100 kDa for *P. antipodarum* (only observed at D14) revealed a pattern similar to that with PBL and with the PBL/ALP ratio (Fig. 6a). At D14, the 100 kDa protein band density was significantly increased by BPA at $100 \mu\text{g/L}$, and by OP at 1 and $10 \mu\text{g/L}$ compared to the controls (Fig. 6a). The 250 kDa protein band density was also significantly increased by BPA at $100 \mu\text{g/L}$, and OP at 1 and $10 \mu\text{g/L}$, and decreased for OP at $100 \mu\text{g/L}$ and TBT at 5 and 50 ng/L compared to the controls (Fig. 6b). For both bands, the acetone solvent vehicle did not cause differences from the control. The 30 kDa band density did not change significantly with any of the three chemicals, despite its specificity to eggs and to the adult stage.

In *V. piscinalis*, significant differences for PBL and ALP only appeared at D14. The levels of PBL were significantly decreased by OP (nominal threshold concentration $3 \mu\text{g/L}$) and TBT at a nominal threshold concentration $\leq 5 \text{ ng/L}$ (Fig. 5d). Levels of ALP were somewhat induced in organisms exposed to OP (nominal threshold concentration of $32 \mu\text{g/L}$) and TBT (nominal threshold concentration $\leq 5 \text{ ng/L}$) (Fig. 5e). The ratio of PBL/ALP followed a pattern similar to that of PBL, and the ratio was reduced by OP and TBT (Fig. 5f). A densitometric analysis revealed a different pattern from that for PBL (Fig. 6c). These differences appeared only at D14, where 300 kDa band proteins were significantly decreased by BPA at 10 and $100 \mu\text{g/L}$, OP at $100 \mu\text{g/L}$, and TBT at 5 and 50 ng/L compared to the controls (Fig. 6c). At D28, values were significantly increased by BPA at $100 \mu\text{g/L}$, the three OP concentrations and TBT at 5 and 500 ng/L compared to the controls (Fig. 6c). No differences were observed between the controls and the solvent controls, but differences were found in the controls between D14 and D28. The 250 kDa band found in the developmental experiments did not appear in the contamination experiments.

The levels of PBL, ALP, PBL/ALP and protein band density (at 30, 100 and 250 kDa for *P. antipodarum* and 300 kDa for *V. piscinalis*) were analyzed by principal component analysis (all data from both exposure periods pooled) (Fig. 7). Protein band density (Vg-like bands) was more closely associated with the levels of extractable lipids (PBL) than with ALP in acetone-fractionated proteins. In *P. antipodarum* (Fig. 7a), the density of the protein band at 250 and 100 kDa was closely related to PBL and to the PBL/ALP ratio, and these were the major components with factorial weights >0.65 . Levels of ALP and proteins were not related to protein band densities although they were principal components. In *V. piscinalis* (Fig. 7b), the protein band density at 300 kDa was closely related to PBL and to the PBL/ALP ratio and these had factorial weights >0.6 . In *P. antipodarum*, the PBL levels were closely related to the 100 kDa protein band density while the 300 kDa band density was closely related to the PBL/ALP ratio. These measurements were also the major factors with factorial weights >0.75 . A correlation analysis (data not shown) showed that, in *P. antipodarum*, both bands (250 and 100 kDa) were significantly correlated with PBL and PBL/ALP, and the 100 kDa band was also correlated to ALP. In *V. piscinalis*, the 300 kDa band was significantly correlated to the three parameters. The variations in PBL, ALP, PBL/ALP, band density and histological observations by the endocrine disruptors used are summarized in Table 1a and b.

4. Discussion

4.1. Detection of Vg-like proteins in three species of gastropods

Several methods have been developed for the determination of Vg in vertebrates (fish), ranging from immunotechniques (enzyme-linked immunosorbent assays, Western blots and

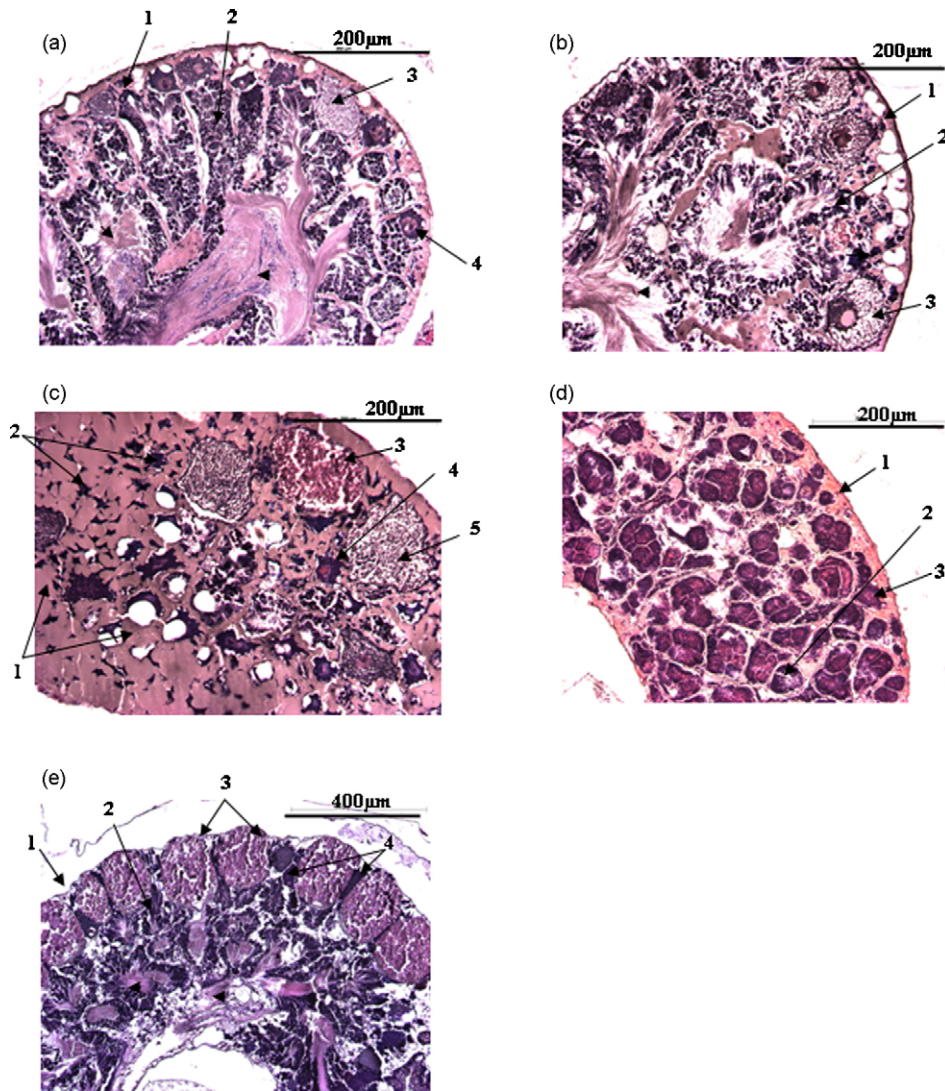


Fig. 4. Histological observations of gonads of *V. piscinalis*. (a) Cross-section of ovotestis of *V. piscinalis* control (20 \times ; thickness 5 μm)—1: germinal epithelium; 2: spermatogonia on sertoli cells; 3: mature oocytes; 4: young oocyte; black arrow: sperm duct. (b) Cross-section of ovotestis of *V. piscinalis* solvent control (20 \times ; thickness 5 μm)—1: germinal epithelium; 2: spermatogonia on sertoli cells; 3: mature oocyte; black arrow: sperm duct. (c) Cross-section of *V. piscinalis* ovotestis exposed to BPA 100 $\mu\text{g/L}$ (20 \times ; thickness 5 μm)—1: inflammatory area; 2: degenerative sperm ducts; 3: mature oocytes; 4: young oocytes; 5: atretic oocytes. (d) Cross-section of *V. piscinalis* ovotestis exposed to OP 100 $\mu\text{g/L}$ (20 \times ; thickness 5 μm)—1: germinal epithelium; 2: degenerative sperm ducts; 3: young oocytes. (e) Cross-section of *V. piscinalis* ovotestis exposed to TBT 500 $\mu\text{g/L}$ (20 \times ; thickness 5 μm)—1: germinal epithelium; 2: spermatogonia on sertoli cells; 3: mature oocytes; 4: young oocyte; black arrow: sperm duct.

immunohistochemistry) to transcript analysis by real-time PCR, DNA hybridization, and protein expression studies (Porte et al., 2006).

The ALP and protein-associated lipids are dependent on the phosphorylation and liposylation states of the egg-specific proteins. The ALP method may therefore not be suited to measuring Vg-like proteins in gastropods, since some other proteins also fractionate in acetone. In this study, we sought to examine Vg-like proteins based on the PBL method for species when the number of phosphorylation sites is close to the mean number of phosphorylation sites of proteins (i.e. background levels). Indeed, as Vgs are lipid- and sugar-rich phosphorylated proteins, Vg assessment based on the lipids/sugars released may be better able to discriminate changes in relative levels of egg-yolk proteins than ALP content, especially in species where Vg-like proteins are less phosphorylated, as it appears to be the case with gastropods (Byrne et al., 1989). This finding was corroborated in the present study, where protein-associated lipids but not ALP are closely associated with the 100-kDa protein band density in *P. antipodarum*. In *L. naticoides*, the PBL/ALP ratio was associated with egg- and female-specific protein band density at

300 kDa. This suggests that the protein-bound lipids procedure could be used to estimate Vg-like proteins in *L. naticoides* (dioecious, sexually dimorphic) and *P. antipodarum* (an ovoviparous parthenogenetic breeder). In *V. piscinalis* (hermaphroditic), where egg extracts were not available for analysis, the PBL/ALP ratio (measured from both adult and juvenile extracts) was closely related to the 300 kDa protein band density profiles, suggesting that this band is associated with the egg-yolk protein precursor. However, this hypothesis will have to be validated by additional studies. These indirect assays are simple, cost-effective biomarkers of endocrine disruption in molluscs and this makes them attractive tools for screening strategies (Matozzo et al., 2008).

In our study, we propose the direct and indirect measurement of Vg-like proteins in three species of freshwater gastropod molluscs, for which neither adapted sequences nor specific antibodies are readily available. Evaluation by the PBL method and the gel electrophoresis method with ultrasensitive silver staining could determine the relative levels of these proteins. Quantitative gel electrophoresis analysis could be more conservative in assessing the relative levels of Vg-like proteins but it is more expensive than the

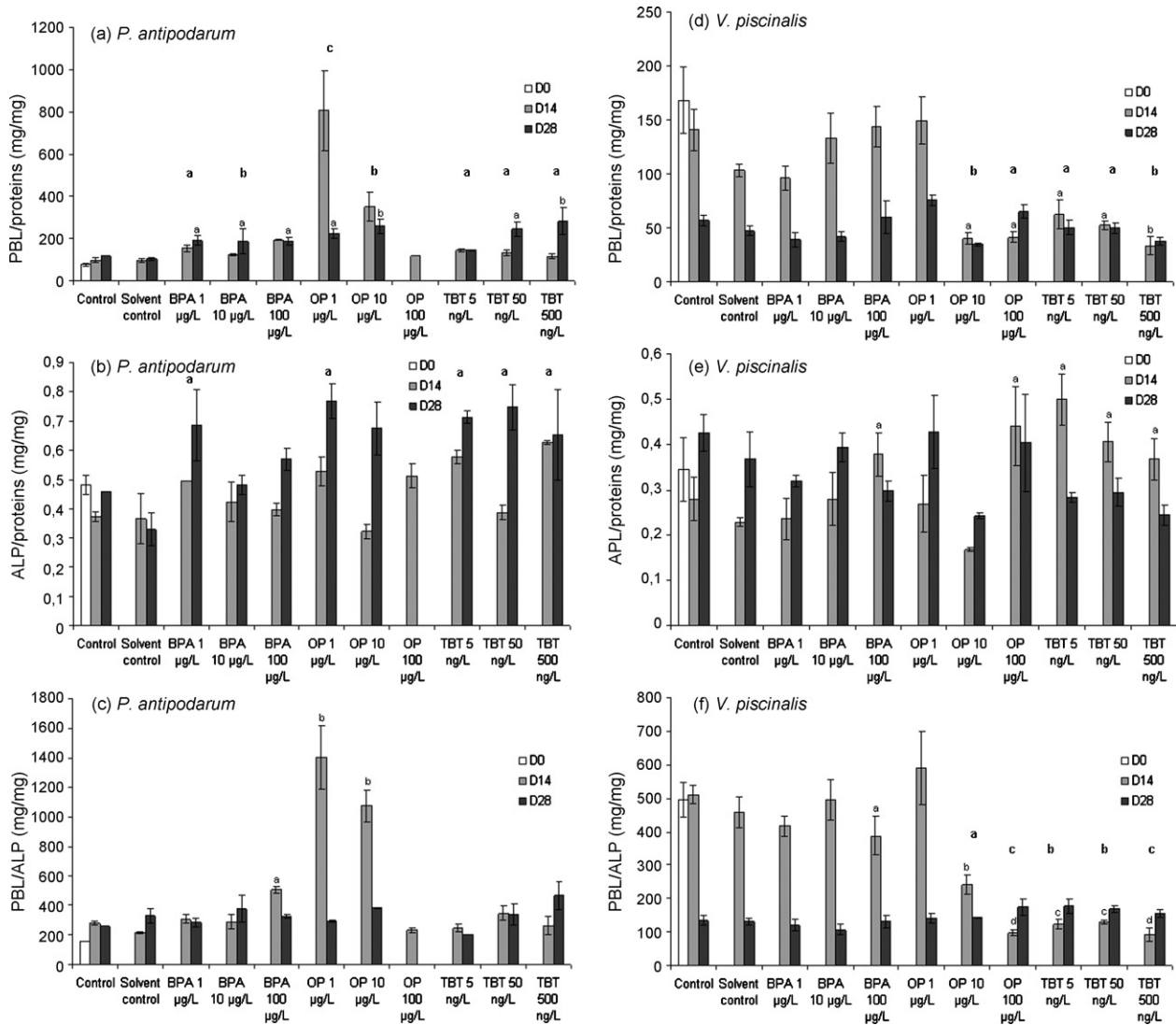


Fig. 5. PBL, ALP (mg/mg proteins) and PBL/ALP (mg/mg) in *P. antipodarum* (a, b, c, respectively) and *V. piscinalis* (d, e, f, respectively) for each pollutant concentration and at D0, D14 and D28. Values are means of three replicates; standard error is presented. ^{a,b,c} $p < 0.01$. $a < b < c$. Bold characters represent significant effects compared to both controls for all times of measurement (D0, D14 and D28). Non-bold characters represent significant effects compared to both controls for a specific time (D14 or D28). $N = 3$ pools of 10 animals.

PBL procedure, which only requires a standard spectrophotometer. The biochemical measurements of ALP and PBL levels, and particularly the ratio between both values, appear to be an interesting parameter for use in discriminating among developmental stages (adult, juvenile or egg) and between the sexes. In order to further support the presence of Vg-like proteins, we also examined the protein profile by gel electrophoresis. Vgs have been identified in the gastropods *Lymnaea stagnalis*, *Helix aspersa*, and *Pomacea canaliculata* (Dreon et al., 2002) as well as in some bivalves (Riffesser and Hock, 2002, Gagné et al., 2005) using this method. In the gastropod *P. canaliculata*, the major egg-yolk protein band eluted at 150 kDa accompanied by the co-migration of bands 67 and 31 kDa, which were considered subunits of the Vg-like proteins. We noted the presence of a similar 30 kDa band in *P. antipodarum*, but the protein band density did not vary with the exposure of the test substances, suggesting that this subunit was not sensitive to these substances (constitutively expressed?). This protein band (30 kDa), however, was readily found in eggs and adults but not in juveniles. In the egg, Vg is proteolytically cleaved into heavy (120 kDa) and light (30–35 kDa) chains: lipovitellins and phosvitins. Apparently,

invertebrate Vg lacks the phosvitin moiety commonly found in vertebrates, thus explaining their lower levels of phosphate content (Byrne et al., 1989). In our study, we found Vg-like protein units at 250–300 kDa for the three species tested and one major protein band (100 kDa); a minor band at 30 kDa was observed in *P. antipodarum*. These bands (250–300 kDa) were the major ones found in the eggs and discriminated well between genders for *L. naticoides*. A hierarchical tree analysis of gel electrophoresis data confirmed that, for *L. naticoides*, the PBL/ALP ratio was closely related to a specific protein band density at 300 kDa. For *P. antipodarum*, the 30 and 100 kDa bands were more closely related to the PBL/ALP ratio than the 250 kDa band. For *V. piscinalis*, the PBL/ALP ratio was closely related to the protein density bands at 300 kDa, but not the 30 kDa protein band. On the whole, these results suggest that Vg-like proteins in these snail species are lipoproteins more than phosphoproteins, thus corroborating previous findings (Byrne et al., 1989; Dreon et al., 2002). Based on an egg-mass analysis, Vg-like proteins appear to exist as subunits in *P. antipodarum* and *L. naticoides*. For *V. piscinalis*, the lack of egg oocytes limits the interpretation of the gel electrophoresis data or of the electrophoregram.

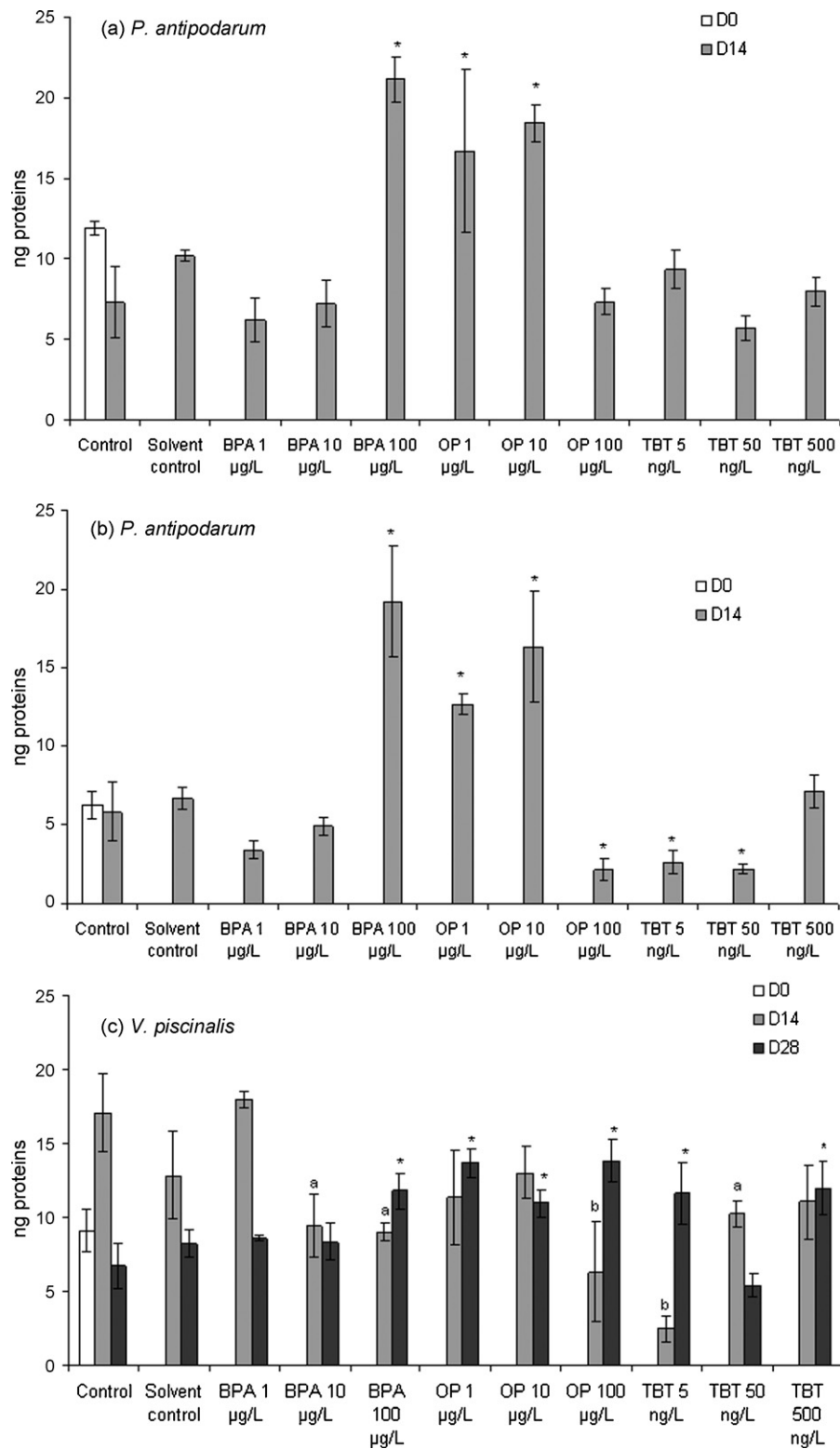


Fig. 6. Quantity of calculated proteins (ng) on 250 kDa (a) and 100 kDa (b) for *P. antipodarum* and 300 kDa (c) for *V. piscinalis* for each pollutant concentration. No analysis was performed on *P. antipodarum* at D28 due to a lack of samples. Values are means of three replicates (three pools of 10 animals); standard error is presented. *Significant difference compared to both controls; $p < 0.05$.

The protein band at 300 kDa was closely related to the PBL/ALP ratio, suggesting that this band might be related to Vg-like protein production. We found that lipoproteins readily precipitated at centrifugation speeds of $10,000 \times g$, indicating their low solubility in homogenates. The protein-bound lipid methodology could be further refined by using a lipophilic fractionation procedure such

as t-butylmethyl ether instead of acetone precipitation (Gagné et al., 2001; Andrew et al., 2008). Proteomic approaches (liquid chromatography and time of flight mass spectrometry) would make it possible to identify specific peptides associated with the lipoprotein family of egg-yolk proteins and the development of specific antibodies or gene expression assays.

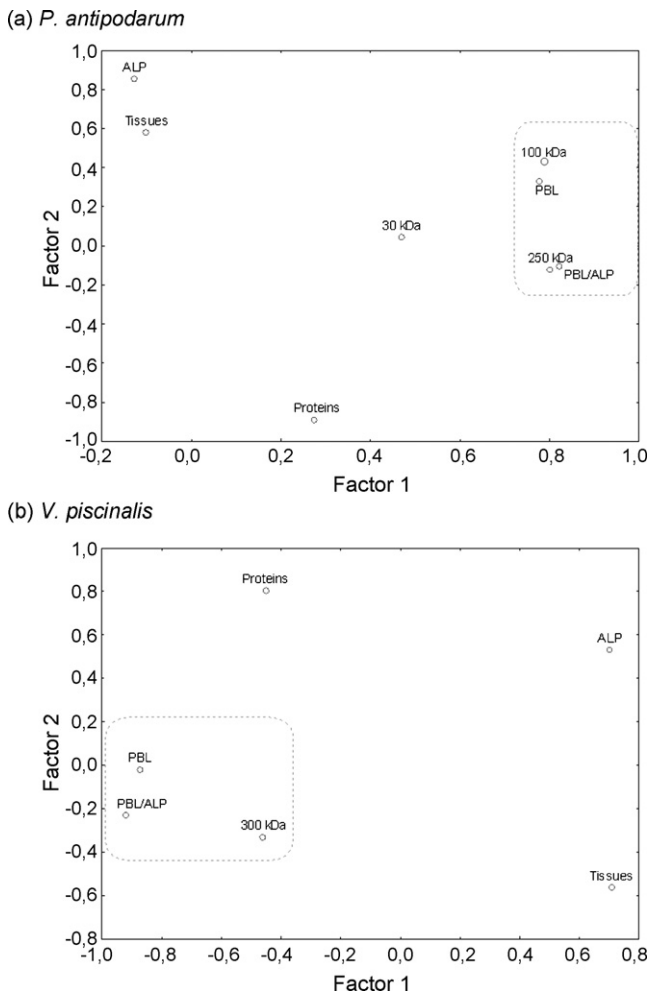


Fig. 7. Factorial analysis of biochemical analyses of egg-yolk proteins in the contamination experiment: (a) *P. antipodarum* and (b) *V. piscinalis*.

4.2. Effects of endocrine disruptors on gastropods

Measured concentrations of TBT were close to nominal levels, while measurements for OP and BPA revealed a decrease of around 50% in accordance with the known behaviour of these compounds in laboratory experiments (Gray et al., 1999; Van den Belt et al., 2001).

Several of the parameters being followed (e.g. PBL, ALP, and band density) were modified by exposure to the endocrine disruptors over the period of the exposure experiment (i.e. on day 14). The lack of response observed on day 28 might have been due to the poor overall health of the organisms (occurrence of mortalities, which could be related to the presence of oligochaetes in the shell of snails).

BPA and OP are known estrogenic compounds in vertebrates, although they are not among the most potent ones. Results of the BPA exposure at high concentrations (>1 mg/L) in *C. carpio* (Mandich et al., 2007) and in Japanese medaka, *Oryzias latipes* (Kang et al., 2002), suggest that BPA is a weak estrogen in fish compared to 17 β -estradiol: BPA alters sexual development and induces vitellogenin in males, but has no adverse effect on their reproductive success. Several studies show the effects of BPA and OP on invertebrate reproduction. A dose- and time-dependent stimulation of embryo production in the brood pouch was shown in *P. antipodarum* after exposure to sediment spiked with OP or BPA at 30 μ g/kg after 2–6 weeks of exposure to BPA and 4–7 weeks to OP (Duft et al., 2003b). Similar reproductive responses were obtained following

exposure to dissolved BPA, OP and EE2 in *P. antipodarum*, stimulating egg/embryo production in the snail at low levels (25 μ g/L) and causing inhibitory effects at higher levels (100 μ g/L) (Jobling et al., 2004). In our experiment, we found no modifications in fecundity, regardless of the species (number of juveniles or embryos for *P. antipodarum* and number of egg mass for *V. piscinalis*). However, the exposure period (i.e. four weeks) was short compared to laboratory studies showing reduced fecundity in these species (Duft et al., 2003b; Jobling et al., 2004) and may be too short to produce an effect in these parameters at the test concentrations.

Morphological and histological changes can highlight endocrine-disrupting effects (Ketata et al., 2008). In the prosobranchs *Nucella lapillus* and *M. cornuarietis* exposed to OP, BPA or EE2, enlargement of accessory pallial sex glands, formation of additional female organs, massive dose-dependent stimulation of oocyte production and high mortality in females as well as reduced penis and prostate gland length in males were shown (at 1 μ g/L of OP or BPA and 1 ng/L EE2) (Oehlmann et al., 2000; Oehlmann and Schulte-Oehlmann, 2003). This syndrome is described as “superfeminization”. Interestingly, 100 μ g BPA/L then led to a “paradoxal imposex” phenotype, considering that BPA is an estrogenic compound (Oehlmann et al., 2000). In *M. edulis* exposed to BPA, animals had almost all ovarian follicles filled with atretic oocytes compared to the controls, which contained mature oocytes (Aarab et al., 2006). Another experiment on the same species also showed gamete resorption in both sexes and damage to ovarian follicles and oocytes (Ortiz-Zarragoitia and Cajaraville, 2006). In our experiments, we found that BPA and OP induced a similar effect in the hermaphrodite *V. piscinalis* by completely dismantling the male parts of the gonad, while no effect was noticed on *P. antipodarum*, which only possesses female reproductive parts. In our experimental conditions, we also observed some impairment of female parts on the ovotestis of *V. piscinalis*, with the apparition of degenerative mature oocytes for BPA and the presence of young oocytes for OP only. These results suggest that BPA and OP may disturb the molluscan reproductive and endocrine system, although the underlying mechanism by which this occurs is still unknown. These results also support the hypothesis that effects on the macroscopic traits of reproduction (number of eggs) can be detected after longer exposure periods.

During synthesis, Vg undergoes substantial post-translational modifications, including phosphorylation, glycosylation, cation and lipid mobilization, all of which are altered in the presence of contaminants (Gagné et al., 2002) and are altered further during fertilization and early embryogenesis. The effects of BPA on Vg-like protein levels reported in the literature are contradictory: in one experiment, the exposure of blue mussels to 50 μ g/L BPA caused no changes in Vg protein content in either males or females (Ortiz-Zarragoitia and Cajaraville, 2006); in another experiment, the same exposure in the same species was shown to induce the expression of Vg proteins in females (Aarab et al., 2006). OP (10–100 μ g/L) did not modify plasma Vg or hatching rates in zebrafish, *Danio rerio* (Van den Belt et al., 2001). However, BPA and OP induced plasma Vg synthesis in fathead minnow, *Pimephales promelas*, rainbow trout, *Oncorhynchus mykiss*, and *C. carpio*, stimulating Vg at low levels (<25 μ g/L) and causing inhibitory effects at higher levels (100 μ g/L) (Jobling et al., 2004). In our study, in *P. antipodarum*, PBL/ALP ratios and protein quantities of the 250 and 100 kDa bands assimilated to Vg-like proteins were increased by high concentrations of BPA and low concentrations of OP; moreover, Vg-like protein quantity was reduced by the highest concentration of OP (100 μ g/L), showing an inhibitory effect at high levels. The finding of Jobling et al. (2004) corroborates our own, which were obtained with direct and indirect measurements (PBL/ALP and GE) for Vg-like protein. The results are less clear for the hermaphroditic *V. piscinalis*: the PBL/ALP ratio

Table 1

Synthesis of results obtained in the contamination experiment performed in this study for the different endpoints measured.

(a) <i>P. antipodarum</i>		PBL D28		ALP both times pooled		PBL/ALP D14		Band density D14			
BPA											
1 µg/L		↑		↑							
10 µg/L		↑									
100 µg/L		↑				↑		↑ 250 and 100 kDa			
OP											
1 µg/L		↑		↑		↑		↑ 250 and 100 kDa			
10 µg/L		↑				↑		↑ 250 and 100 kDa			
100 µg/L								↓ 100 kDa			
TBT											
5 ng/L				↑				↓ 100 kDa			
50 ng/L		↑		↑				↓ 100 kDa			
500 ng/L		↑		↑							
(b) <i>V. piscinalis</i>		PBL D14		ALP D14		PBL/ALP D14		Band density		Histology (D28 only)	
								D14		D28	
BPA											
1 µg/L								↓			
10 µg/L								↓		↑	
100 µg/L		↑		↓		↓		↓		↑	
OP											
1 µg/L								↑			
10 µg/L		↓				↓		↑		↑	
100 µg/L		↓		↑		↓		↑		↑	
TBT											
5 ng/L		↓		↑		↓		↓		↑	
50 ng/L		↓		↑		↓		↓			
500 ng/L		↓		↑		↓		↑			

Arrows represent significant trends observed compared to controls and solvent controls (no difference was observed between the controls). (a) *P. antipodarum* (results at D28 for PBL, both time periods (data pooled) for ALP and at D14 for PBL/ALP and band density). (b) *V. piscinalis* (results at D14 for PBL, ALP and PBL/ALP, at D28 for histology, and at D14 and D28 for band density).

decreased for high concentrations of BPA and OP, and the Vg-like protein quantity showed different patterns of responses, depending on the time of measurement (decreasing at 14 days and increasing at 28 days). The decrease was apparently the result of the transient increase in ALP levels. It should be borne in mind that *V. piscinalis* is hermaphroditic and thus the effects of oestrogenic compounds are mitigated by the gametogenetic stage and whatever fertilization processes might have been occurring. Further experiments will be required to elucidate the effects of xenoestrogens on the production of Vg-like proteins, especially at the early gametogenetic stages.

Imposex is defined as a virilization phenomenon or a pseudo-hermaphroditic condition characterized by the development and superimposition of non-functional male sexual characteristics (a penial vas deferens and/or seminiferous tubules) in female individuals. This phenomenon has been described in more than 150 species of gastropods from different geographic regions, including *N. lapillus*, *Ilyanassa obsoleta*, *Nassarius reticulatus* and *M. cornuarietis* (Matthiessen et al., 1999). Imposex can be induced at 1 ng Sn L⁻¹ (Alzieu, 2000), which is lower than the first TBT concentration used in this study. Gastropod species that have low fecundity or limited mobility and hatchlings that are not widely dispersed suffer from the reduction or elimination of populations in severely affected locations (Matthiessen et al., 1999). The mechanism of imposex by TBT is still under debate and has been attributed, in part at least, to elevated testosterone levels in female gastropods resulting from the inhibition by TBT of the cytochrome P450 aromatase, which catalyses the conversion of testosterone into estradiol (Sponner et al., 1991; Bettin et al., 1996). Another mechanism underlying imposex implicates the retinoid receptor pathway, where both TBT and retinoic acid significantly increase penis length in dodwelks (Castro et al., 2007). We observed no histological modifications in any of the gastropod species used in this study. However, not all prosobranch

species are susceptible to imposex and interspecies differences in susceptibility remain poorly understood. The female periwinkle *Littorina littorea* does not develop a penis in the presence of TBT, but does show, at high TBT concentrations (5–10 ng/L Sn), a transformation of its pallial organs toward a male morphological structure (development of a prostate gland). This phenomenon is termed *intersex* (Matthiessen and Gibbs, 1998). In our study, we showed no development of a male structure, but we did find that gonads of *V. piscinalis* exposed to TBT had an elevated number of mature oocytes compared to the controls. In *P. promelas*, a steroid metabolism inhibitor (fadrozole) was shown to shut down spawning by preventing maturation of oocytes (Leino et al., 2005). One hypothesis says that TBT disturbs the normal development and expulsion of oocytes. This hypothesis is supported by the fact that ALP levels were increased by TBT in *V. piscinalis* (Fig. 5), which could be an indirect effect of TBT. The increase in ALP in TBT-exposed snails might result from the arrest of oocyte maturation. Oocyte maturation involves the activation of protein kinases such as p34(cdc2) (Nagahama and Yamashita, 2008), where the arrest of maturation (inactivation of protein kinases) could result in the high phosphorylation of proteins. Interestingly, the maturity-inducing hormone 17 α ,20 β -dihydroxyprogesterone, induces the overexpression of a 30 kDa protein, which was identified to be cyclin B (Bhattacharya et al., 2007). Further work is warranted to determine whether the 30 kDa protein is from the cyclin B family in *V. piscinalis*. Densitometric analysis of the 30 kDa protein band did not significantly change relative to the controls but the phosphorylation state was not determined in the present study.

Besides imposex, TBT has been shown to induce other effects on molluscs, especially fecundity, at 7–12-week exposure periods (Czech et al., 2001; Duft et al., 2003a; Leung et al., 2007). As with BPA and OP, we found no effect of TBT on fecundity in the two species studied. We can hypothesize that the exposure period (i.e. four

weeks) may be too brief to see any effect of TBT on these parameters at the test concentrations.

In our experiment, TBT did not significantly modify the PBL/ALP ratio in *P. antipodarum* but decreased Vg-like protein quantities (of the 250 kDa band) at 5 and 50 ng/L, with no effect on the female and egg-specific protein band at 100 kDa. The three concentrations of TBT also decreased the PBL/ALP ratios for *V. piscinalis*. This decrease in Vg-like proteins by TBT was reported in a study of *M. arenaria* exposed to harbour water contaminated with TBT (Gagné et al., 2003). Some studies on TBT were conducted on Vg fishes: TBT decreased Vg-mRNA expression in Atlantic salmon, *Salmo salar* (Mortensen and Arukwe, 2007) and also decreased ALP in female of the false kelpfish, *Sebastes marmoratus* (Zheng et al., 2006). As TBT reduces estradiol synthesis by blocking cytochrome P450 aromatase, we could hypothesize that TBT could reduce vitellogenin levels. Our results could therefore indicate a potential androgenic action of TBT on these gastropods.

5. Conclusion

In conclusion, the levels of Vg-like proteins were determined directly by gel electrophoresis with silver staining and indirectly by alkali-labile phosphates and protein-bound lipids in acetone-fractionated proteins. Since egg-yolk proteins contain few phosphorylation sites, their evaluation by the ALP procedure was compromised. However, the levels of lipids associated with acetone-fractionated proteins were closer to the levels of egg and female-specific proteins in *P. antipodarum* and *L. naticoides*, which showed 100–30 and 300 kDa protein bands, respectively. In the hermaphrodite *V. piscinalis*, the lack of female-specific tissues complicated interpretation of the gels. Exposure to BPA and OP increased the quantity of protein bands at 100 and 250 kDa for *P. antipodarum* on day 14 and increased the 300 kDa protein band on day 28 (but not day 14) for *V. piscinalis*. The levels of PBL and the PBL/ALP ratio were also increased by BPA and OP in *P. antipodarum*, but the increase in *V. piscinalis* was not significant. TBT decreased the levels of the 250 and 100 kDa (but not significantly for the latter) protein bands in *P. antipodarum* and the PBL/ALP ratio in *V. piscinalis*. No effects were shown on fecundity, probably due to the too brief exposure period.

This study represents the first attempt to measure Vg-like proteins in three species of gastropods with differing modes of reproduction. We showed that direct method such as gel electrophoresis and indirect method such as protein-associated lipid/ALP ratio can determine the presence of Vg-like proteins and that these parameters could be useful general indicators of stress. Further investigations with different kind of pollutants (EDs or not) are needed in order to determine if the parameters developed in this study are specific to endocrine disruptors. A next step of our work would be to study the effects of EDs on the parameters developed in the dioecious species, *L. naticoides*, in order to compare results obtained with the two other species. The parameters developed in this study could be useful in field studies, but additional studies remain necessary to carefully assess the range of these responses both in exposed and control organisms as well as the biological significance of the variations on the fecundity.

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