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Impact of cadmium on the ecdysteroids production in *Gammarus fossarum*

Sondes Abidi[1,2] · Khedidja Tair Abbaci[1] · Olivier Geffard[1] · Moncef Boumaiza[2] ·
Adeline Dumet[3] · Jeanne Garric[1] · Nathalie Mondy[3]

Abstract *Gammarus fossarum* is an important test organism which is currently used as a bio-indicator as well as in ecotoxicological tests. Nevertheless, data on ecdysteroids in endocrine toxicity test are not yet available for these species, despite its crucial role in molting and reproduction. In the present paper, ecdysteroids concentrations were studied during the molt cycle (in females) and embryonic development in *G. fossarum* (Crustacea, Amphipoda) in order to propose an ecdysteroids toxicity test. Ecdysteroids levels in *G. fossarum* showed a single peak during premolt at stage Dl-D2. In embryos, ecdysteroids levels progressively increased over stages 3 and 4, with peak levels at stage 4. A Cadmium toxicity test was proposed to examine if the molting and embryogenesis disturbances previously observed after cadmium exposure (Geffard et al. 2010) could be attributed to changes in ecdysteroids titers. Exposure to the different cadmium concentrations (3; 9; 300; 900 µg/l) increased ecdysteroids secretion by Y-organs in vitro, but it had no significant effect on exposed embryos (in vivo). Based on previous findings, we are led to conclude that the molting impairments in cadmium-exposed females of *G. fossarum* is connected to the changes in ecdysteroids concentrations.

Introduction

The need for new and updated test methods to detect and characterize endocrine disrupting chemicals has been expressed by the Task Force on Endocrine Disrupters Testing and Assessment (EDTA). At the early meetings of EDTA Task Force, there appeared that existing Endocrine Disrupter Testing and Assessment (OECD) Test Guidelines would insufficiently cover for endocrine-related effects, especially for the environment.

Member countries decided to list test methods which could potentially (i) cover the effects of chemicals on the reproductive system and on the development (ii) proposed enhancements where needed. Invertebrate life-cycle tests, encompassing development and reproduction, are proposed as in vivo assays including endpoints that indicate mechanisms of adverse effects (endocrine and other mechanisms) and potential population damage (OECD 2012). Several factors highlight the interest of those organisms in endocrine disruption studies: (i) they are important in routine ecotoxicological testing and environmental monitoring (ii) numerous invertebrates are highly valued by humans for commercial purposes (iii) many of them have key positions in aquatic and terrestrial food webs. Therefore, a great effort has been invested into improving our understanding of invertebrate endocrine systems and their potential chemical disruption. In 2000, Lafont described and gave examples of four levels at which endocrine

✉ Sondes Abidi
abidi.sondes@yahoo.fr

[1] Milieux aquatiques, Ecologie et pollutions. Equipe Ecotox, irstea, Lyon, Villeurbanne, France

[2] Present Address: Unité d'hydrobiologie Littorale et Limnique, Laboratoire de Bio-surveillance de l'environnement, Faculté des Sciences de Bizerte, 7021 Jarzouna, Tunisia

[3] Laboratoire d'Ecologie des Hydrosystèmes Naturels et Anthropisés, Université Lyon 1 43, Bd du 11 novembre 1918, UMR 5023, Bât Darwin C étage 2, 69622 Villeurbanne Cedex, France

disruptors (EDCs) can disturb endocrine systems. At the first levels, EDCs block the availability of the precursors required for the synthesis of hormones. The interruption of hormone biosynthesis occurs at the second levels. For example, some chemicals inhibit cytochrome P450 s which catalyzed biosynthesis, thus breaking the synthetic pathway leading to hormone production (Lafont 2000). The third levels at which EDC can act is on hormone catabolic processes. In this case, EDC can act to increase the rate at which a hormone is catabolized, resulting in lower levels in an animal. Then, at the fourth levels, EDC directly interferes with the actions of hormones. EDCs can act as agonists by binding to a hormone receptor. Therefore, it would regulate gene transcription (LeBlanc et al. 1999). EDC also may act antagonistically by binding to a hormone receptor without inducing its activity (LeBlanc et al. 1999).

According to Soin et al. (2009), ecdysteroids, key hormone in molting and reproduction (Subramoniam, 2000) has turned out to be an important target for endocrine disruption research over the past decade. Endocrine disrupting chemicals may interfere with hormonal signaling by binding directly with the ecdysone receptor and acting as an agonist or antagonist (LeBlanc 2007; Mu and Le Blanc 2004; Rodríguez et al. 2007; Williams et al. 2000). Alternatively, some chemicals can elicit anti-hormonal effects by interfering with the synthesis of the hormone. The inhibitors of cytochrome P450 enzymes disrupt steroid hormone synthesis, leading to reduced hormone levels and associated endocrine toxicity (Mu and Le Blanc 2004).

Cadmium is known to affect *Gammarus fossarum* molting, vitellogenesis and embryogenesis (Geffard et al. 2010). Cadmium exposure (3 µg/l) inhibits feeding rate and vitellogenesis together with an apparition of abnormalities in embryos. Moreover, the intermolt period becomes longer: the percentages of females in stage C2 decrease and 70 % of females reveal C1 at molt stage. These observations indicate that the deleterious effects of cadmium on reproduction, molt and embryogenesis may be the result of its direct interference with metabolism or the mechanism of action of ecdysteroids.

The present study aims to characterize the pattern of ecdysteroids in female *G. fossarum* and their embryos in order to investigate if cadmium exposure affects *Gammarus* ecdysteroids titers. Such experiments are expected to provide explanations to the observations by Geffard et al. (2010). We would first describe female ecdysteroids levels through molt stage and then analyze them during embryonic development. Subsequently, for the sake of figuring out the Cd mechanism on ecdysteroids in both females and embryos, we have decided to speculate the in vitro effect of cadmium on the production of these hormones in the molting gland. However, this experiment was difficult for embryos because they are small and the

dissection of Y-organs was hard. Therefore, we examined the effect of in vivo exposition on the embryonic ecdysteroids rates.

Materials and methods

Determination of the ecdysteroids levels in females and embryos of *G. fossarum*

Ten stages (A; B; B–C1; C1; C1–C2; C2; C2–D1; D1; D1–D2; D2) of ovigerous females have considered for the analysis of ecdysteroids levels during a molting cycle. In order to avoid the confusion between the maternal and embryonic ecdysteroids, embryos were removed from the marsupium of each female. The molting stage identification was determined according to criteria proposed by Geffard et al. (2010). The molt cycle is divided in three main periods, post-, inter- and pre-molt stages. These phases were respectively characterized by the hardening of the new cuticle, the retraction of the epidermis from the old cuticle and by the secretion of the new cuticle. For animal staging, the ends of the 3rd and 4th pairs of pereiopods (dactylopodite and protopodite) were sectioned at the levels of the carpopodite by Wecker scissors and then mounted between a slide and a cover slip. The carpopodite evolution and the protopodite tissues are observed using the ×630 magnification microscope DM 2500 ® (Leica). The criteria of each molt stage are given in Table 1.

To reveal the possible relationship between oogenesis and the ecdysteroids in *G. fossarum*, we relied on the results obtained by Geffard et al. (2010) concerning the evolution of the mean follicle surface in relation to molt cycle (stages: B, C1, C2 and D1).

Nine stages of embryonic development were used (1a, 1b, 1–2, 2, 2–3, 3, 3–4, 4, 4–5). These embryos were manually recovered from the marsupium with a fine forceps under a binocular loupe. Then, they were placed on a water slide and observed under a binocular microscope (Geffard et al. 2010). In stage 1a, embryos were newly fertilized, oval, and undifferentiated eggs. During Stage 1b, the volume of eggs increases. In stage 2, embryos were characterized by a comma-like shape. Stage 3 was identified by the presence of cephalothorax and segmented appendages. At the next stage (4), the compound eye was fully visible and appendages were also fully developed. The last stage (5) corresponds to newly hatched juveniles (Geffard et al. 2010).

Cd exposure

For the sake of figuring out the Cd mechanism on ecdysteroids in both females and embryos, we have decided to

Table 1 Different main molting stages in *G.fossarum* (A, B, C1, C2, D1 and D2)

Molting stage	Characters
A	The new cuticule is fine and soft
	The tissue is uniformly filled with lacune and haemocytes
B	The harding of the cuticule is observed at dactylopodite setae level
C1	Epidermal retraction at setae level and along the protopodite
C2	The new dactylopodite was retracted and epidermis is inverted at the base of it.
D1	Cuticule was observed on the new dactylopodite
D2	New cuticule also appeared on the new setae of protopodite

Intermediate stages (B–C1; C1–C2; C2–D1 and D1–D2) are identified by common characters between two main stages

speculate the in vitro effect of cadmium on the production of hormones in Y-organ. However, this experiment was difficult for embryos because they are small and the dissection of Y-organs was hard. Therefore, we examined the effect of in vivo exposition on the embryonic ecdysteroids rates.

In vitro Cd-exposure of Y-organ

The M199 culture medium was selected for this experiment because it does not interact with ecdysteroids enzymo-immunoassay (EIA) and has successfully been used in the Y-organ culture (Toullec 1999). Fetal serum calf (10 %) and antibiotics (streptomycin and penicillin) were added and the medium was then adjusted to the *G. fossarum* osmolarity (≈ 40 mosmol/L) (Toullec 1999). From the solution of culture medium with a concentration of 3 mg/l in Cd, low concentrations were prepared (0 for control, 3; 9; 90; 900 μg/l; V = 10 ml each).

Females of stage D1–D2 were sampled for Y-organ-isolation. In this stage, we obtained a high ecdysteroids secretion by the molting gland. Then, in the cell culture plate, we put the culture medium containing cadmium. For each Cd-concentration, we made three replicates and in each well we put 5 Y-organs. Incubation was maintained at 16 °C during 48 h and the samples were then stored at −20° C until use.

Embryos Cd-exposure

According to the literature review (Blanchet et al. 1976; Graf and Delbecque 1987), a high amount of ecdysteroids should be present before hatching. Accordingly, we used stage 4 embryos for experiment. 24 h semi-static Cd-exposure tests were conducted at a temperature of 12 °C and a conductivity of 600 ± 50 μS/cm. Four concentrations of Cd (3; 9; 90 and 900 μg/l) were studied and water control without toxicant was included. For each condition test, four replicates of 11 or 16 embryos were exposed in 500 ml glass beaker. At the end of the experiment, embryos were preserved at −20 °C for the ecdysteroids titers.

Determination of ecdysteroids levels by the EIA (enzyme immunoassay) technique

The techniques used to determine the produced ecdysteroids were made using the EIA procedure described by Maniere et al. (2004) and adapted to gammarids in the UMR 5023 of the University of Lyon 1. This method is based on a competition between ecdysteroids and a tracer (2-succinyl-20-hydroxyecdysone bound to peroxidase) for anti-ecdysteroids antibodies. A L2 polyclonal antibody (gift from Dr. Delbecque, University of Bordeaux, France) is very sensitive to ecdysone, 2-deoxyecdysone and 3-dehydroecdysone, but six times less sensitive to 20-hydroxy-ecdysone (20HE). Each analyzed sample was measured at least in duplicate. 20HE was used for references curves, ecdysteroids titers are expressed in 20HE equivalents (Mondy and Corio-Costet 2000).

Statistical studies

Statistical tests were carried out in order to (i) compare the ecdysteroids amounts during different molting and embryonic stages (ii) show if there is a significant effect of Cd on ecdysteroids concentrations. All results are expressed as mean ± standard deviation. The normal distribution of the variables and the variance homogeneity were tested using the Shapiro–Wilk test and Hartley–Cochran–Bartlett tests, respectively. Differences between groups of gammarids were evaluated by post hoc comparisons were performed using Turkey's test. Relationships between variables were examined by regression analysis. The significance *p*-levels was set at 0.05. These analyses were carried out with STATISTICA® software v 9.0 (Statsoft).

Results

The ecdysteroids levels in female *G. fossarum* and its relationship with oogenesis

Figure 1 shows that during stages A; B; B–C1; C1 and C1–C2, ecdysteroids levels are low and vary between 1.20 and
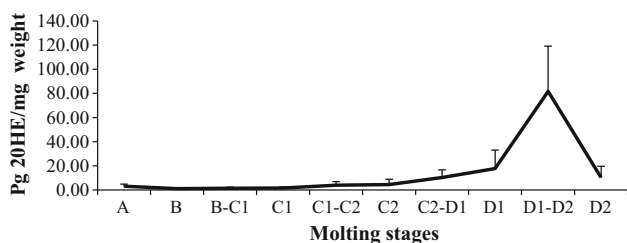
Fig. 1 Amounts of ecdysteroids (pg 20HE/mg weight) detected by polyclonal antibody, in females of *G. fossarum* during molting stages. Data are expressed as means ± sd based on four replicates of 10 females each. A large peak of ecdysteroids was observed just before ecdysis. (A B B–C1 C1 C1–C2) entre 1.20 et 5

4 µg/l. Throughout stages (C2; C2–D1 and D1), the individual titers of ecdysteroids increase and range from 5 to a maximum of 18 ± 5 pg 20HE/mg fresh weight. A sharp peak is then observed during late Dl–D2 with individual titers reaching a maximum of 82 ± 37 pg 20HE/mg fresh weight. At the passage to D2, a rapid decrease of these titers brings them back to the former levels of the pre-molt stages (average: 10.3 ± 5 pg 20HE/mg fresh weight). This variations in ecdysteroids titers are significant (p = 0.0015). During the molt stages A, B, and C, ecdysteroids levels remain low (≤4.5 ± 2 pg 20HE/mg fresh weight).

Concerning the relationship between oogenesis and the ecdysteroids in female *G. fossarum*, our main outcomes emphasize a good correlation (R^2: 0.715) between oogenesis and the amount of ecdysteroids from stage AB to stage D1 (Fig. 2).

Ecdysteroids levels in embryos of *G. fossarum*

During the early stages of embryonic development, the levels of ecdysteroids are low and vary between 1 ± 0.54 pg 20HE/embryo in stage 1 and 4 ± 1.8 pg/embryo in stage 2–3. Over stages 3 and 4, we notice a progressive increase of ecdysteroids levels (48 ± 38 and
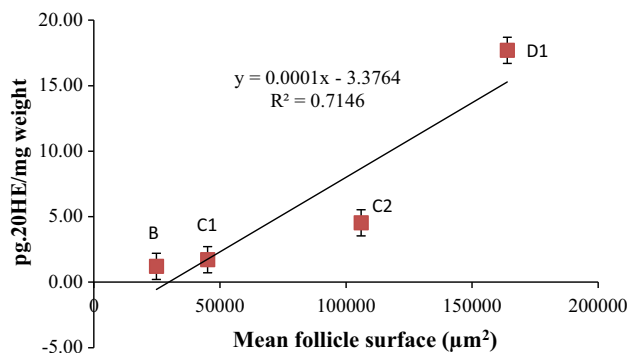
103 pg 20HE/embryo during stage 3 and stage 3–4, respectively, with peak levels at stage 4 (181 ± 64 pg 20HE/embryo). A significant drop (p ≤ 0. 05) of ecdysteroids levels (20.5 ± 16 pg 20HE/embryo) is observed at stage 4–5 (Fig. 3).

Effect of Cd exposure on in vitro ecdysteroids production by the Y-organ

In all cases, after 48 h of Cd exposure, the levels of ecdysteroids are higher than in controls (p ≤ 0. 0002) (Fig. 4).

Effect of Cd exposure on embryonic ecdysteroids

Our results point to the fact that Cd has no significant impact on embryonic ecdysteroids compared to controls (p ≥ 0.05) (Fig. 5).

Discussion

Ecdysteroids in females of *G. fossarum*

The present study describes ecdysteroids levels in *G. fossarum* (females and embryos) with the aim of developing a test of endocrine toxicity. In *Gammarus* females, ecdysteroids follow the general pattern described for other crustaceans (Chang and Mykles 2011; Cuzin-roudy et al.1989; Fingerman 1987; Hyne 2011; Subramoniam 2000). However, they do not show the transitory peak (in stage B) found in the majority of other crustaceans e.g. *Palaemon. serratus* (Baldaia, et al. 1984); *Orchestia gammarella* (Blanchet et al. 1976) or *Orconectes limosus* (Willig and Keller 1973). According to Baldaia et al. (1984), this small peak is concomitant with the post-ecdysial processes of cuticular synthesis (i.e., with the
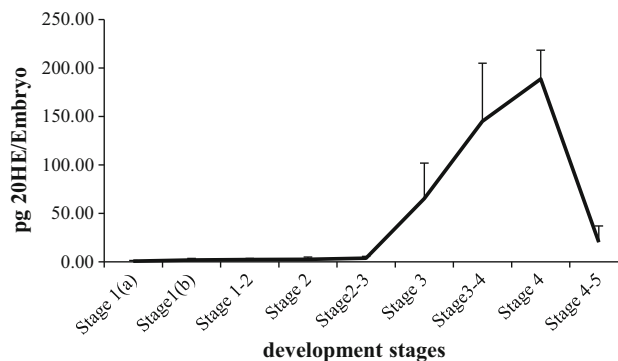

Fig. 2 Correlation between mean follicle surface (μm^2) and amount of ecdysteroids (pg 20HE/mg weight) during four molting stages (B, C1, C2, D1) in female of *G.fossarum*

Fig. 3 Ecdysteroids amounts (pg 20HE/Embryo) detected by polyclonal antibody, in embryos of *G.fossarum* during development stages. Data are expressed as mean ± sd based on four replicates of 13 embryos each. A peak of ecdysteroids was observed on stage 4
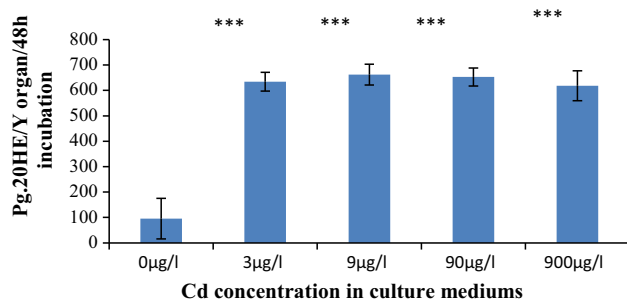
Fig. 4 Amounts of ecdysteroids releasing by Y-organ of *G.fossarum* in culture medium after a 48-h exposure to several increasing cadmium levels (3; 9; 90; 900 μg/l). Data expressed as mean ± sd based on four replicates of 3 wells each. Significant differences against the controls (0 μg/l) are indicated by asterisks (one way-ANOVA and HSD Tukey post hoc; ($n = 3$; ***$p < 0.0002$)
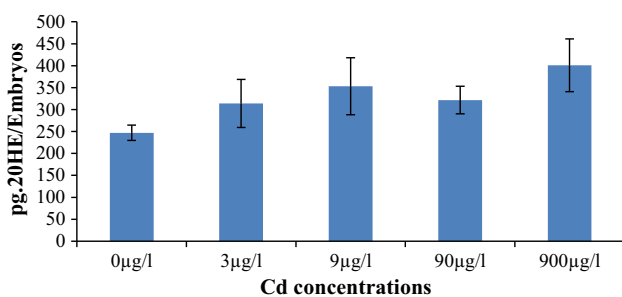

Fig. 5 Amount of ecdysteroids detected by plyclonal antibodies in embryos of *G.fossarum* after a 24-h exposure to several increasing cadmium levels (3; 9; 90 μg/l = 900 μg/l). Data expressed as mean ± sd based on four replicates of 11-16 embryos each

deposition of the main membrane layers). It could alternatively be related to the initiation of premolt (Girard and Maissiat 1983; Hopkins 1983). Graf and Delbecque (1987) emphasize on the importance of a precise molt staging and a determined time of apolysis to get the two peaks. In *G. fossarum*, the evolution of the carpopodite and the protopodite tissues are synchronous with molt and it is easy to exactly define the molting stage during premolt. However, this peak could happen during the intermediate stage A–B which we do not consider in our study.

Our observations of *G. fossarum* unfold that the peak in stage D takes place precisely at D1–D2. It is relatively sharp and ecdysteroids concentrations are significantly reduced in D2. This timing is in good agreement with observations in *Orconectes sanborni* (Stevenson et al.1979) and *Orchestia cavimana* (Graf and Delbecque 1987) for which the peak is situated in Dl–D2 and *Orconectes limosus* (Jegla et al.1983) when it takes place at the beginning of D2.

Ecdysteroids and molt preparation

Our outcomes suggest that in *G. fossarum*, ecdysteroid peak occurs during late Dl–D2 which corresponds to the onset of

epicuticle deposition (Geffard et al. 2010). Therefore, a new cuticle also appears on the new protopodite setae leading up to the conclusion that this peak really triggers the primary steps of cuticulogenesis, i.e., the epicuticle deposition. According to Willig and Keller (1973) the peak observed at stage D is related to the activation of the biosynthetic process during the new cuticle formation. At this stage, the enzymes needed for the development of the new cuticle and the cuticular proteins are synthesized.

Once triggered, cuticle secretion in female *G. fossarum* is preceded by low ecdysteroids titers during the end of premolt (D2) as well as post-molt (stage A and B). Later steps of cuticulogenesis (the hardening of the cuticle) occur during the titer's decrease. After hormonal stimulation, high levels are no longer necessary for cuticle synthesis. The intermolt stage (C) is visible from day 4 and is characterized by epidermal retraction and a progressive little increase of ecdysteroids. It obviously appears that in *G. fossarum*, apolysis occurs at low levels of ecdysteroids several days before the peak.

Ecdysteroids and vitellogenesis

In *G. fossarum*, vitellogenin appears in a low concentration during the post-molt stage (Geffard et al. 2010). Its levels increases throughout the intermolt until D premolt stage and then decreases after exuviations. In our results, we notice a good correlation between oogenesis and the amounts of ecdysteroids from stage AB to stage D1: vitellogenin starts in stage A (fresh molt) when there is a small and sharp rise in the hemolymph ecdysteroids. In the stages B and C, it is developed quickly and the yolk vesicles increased in number and size, the ecdysteroids concentration rises continuously. From stage C2–D1, vitellogenin levels seem to elevate attending maxima at stage D1–D2 (full maturity of the oocytes/before and following the liberation of brood respectively). From these results, we suggest a possible role of ecdysteroids in the female reproductive cycle. These results are also observed in the freshwater prawn *Macrobrachium nipponens*.

According to Okumura et al. (1992), a close interaction between the hemolymph ecdysteroids titers and the corresponding ovarian maturation stages during a reproductive molt cycle is registered.

Levels of ecdysteroids in embryos of *G. fossarum*

In embryos, we observed a very little ecdysteroids which begin to increase during the course of embryonic development to reach a maximum in the pre-hatching stage. This state is also observed in the *Sicyonia ingenti* (Chang et al. 1992) and *P. serratus* (Spindler et al. 1987). Apparently, the presence of ecdysteroids in early embryonic

development stage of *G. fossarum* (1 and 2) has maternal origin. Thus, Geffard et al. (2010) highlights that in the first and second stage, embryos are oval and undifferentiated. Our proposal is consistent with several authors (Hagedorn 1985; Hoffmann and Lagueux 1985; Hyne 2011; Lafont et al. 2005: Lagueux et al. 1984; Rees and Isaac 1984; Thompson and Houk 1987). According to Chávez et al. (2000), maternal ecdysteroids, which have occurred in the early stage of embryogenesis, regulate morphogenetic processes such as cell movements and cuticle deposition in embryos. In many insect species, ecdysteroids are synthesized in developing ovaries, accumulated in mature ones and are then transferred to eggs.

Ecdysteroids amount during embryonic development indicates that ecdysteroids levels are progressively increased over stages 3 and 4, with peak levels at stage 4. The increase of ecdysteroids during stages 3 and 3–4 may be correlated to the cephalothorax apparition, the development of appendages and compound eye as observed by Geffard et al. (2010). The major peak observed at the pre-hatching stage (stage 4) may be correlated with the deposition of the embryonic cuticle. At stage 5, there is a drop in ecdysteroids. As a result, the molting glands release a large amount of them during the previous stages.

Effect of Cd on ecdysteroids production

A clear result obtained from Geffard et al. (2010) was the significant inhibition of molting in *G. fossarum* exposed to 3 μg/l of cadmium. In Crustacea, It has been strongly suggested that the development is controlled by circulating ecdysteroids (Vight and Fingerman 1985). Also, in recent years, an increasing number of studies have investigated the potential interference of chemicals with ecdysteroids signaling in crustaceans-dependant anti-ecdysteroidal activity in daphnids by lowering endogenous ecdysteroid level and delaying molting (Mu and LeBlanc 2002). In the latter study, developmental abnormalities in exposed daphnid embryos were associated with suppressed ecdysone levels that could be prevented by co-exposure to 20-hydroxyecdysone. Additonal studies have demonstrated that several other compound cause adverse effects in crustaceans that are consistent with possible anti-ecdysteroidal activity including 4-nonylphenol (Shurin and Dodson 1997), propiconazole (Kast-Hutcheson et al. 2001) Cadmium (Abigail et al. 2003) and Buprofezin (Kobayashi et al. 1989). These results clearly demonstrate that ecdysteroids are critical to normal crustacean development and that environmental chemicals which have the potential to interfere with ecdysteroids signaling can cause significant developmental effects. Therefore, we have suggested that the molting inhibition exerted by cadmium is rather related to an effect of cadmium on the secretion of ecdysteroids

from Y-organ, since our results showed that, the hemolymphatic ecdysteroid levels correlated well with the molting stage.

The *G. fossarum* Y-organ toxicity test allows possible an examination of the direct effect of metal on ecdysteroids production. The test duration and methodology are selected to be the shortest as possible, so that it indicates the Cd action on ecdysteroids. In vitro exposure of Y-organ to different cadmium levels leads to a significant stimulation of ecdysteroids production. We suggest a direct stimulation of cadmium on the enzymes responsible for ecdysteroid synthesis in the Y-organ.

Other studies have revealed the stimulatory effects of cadmium on the steroid hormone concentrations in aquatic organisms. In cadmium-exposed fish, the levels of both steroids are detected to be higher than in controls (Sangalang and Freeman 1974). In addition, testosterone and progesterone levels in the several organs of starfish, that are exposed cadmium for 3 weeks, are significantly higher than the corresponding values in controls (Voogt et al. 1987). The administration of Cd to *Daphnia magna* (Bodar et al. 1990) does not simply rise as a result of a decline in bodyweight, but also for the overall significant stimulatory effect on ecdysteroids production.

Cadmium induces abnormal levels of Y-organ ecdysteroids secretion. This observation might have been the primary cause of molting (direct effect) and reproduction disturbances (indirect effect) observed in previous cadmium toxicity studies (Geffard et al. 2010). Engel and Brouwer (1987) suggest interrelationships between ecdysteroids, metallothioneins and metallo-enzymes during the molt cycle of the blue crab *Callinectes sapidus*. Under unstressed conditions, zinc metallothioneins and ecdysteroids interact to affect, for instance, the zinc-dependent carbonic anhydrase activity. If similar mechanisms are active in *Gammarus*, accumulated cadmium will most probably interfere with this essential metal-regulatory system. This in turn will have considerable implications on the molting process, ovogenesis. This hypothesis is attractive since the results of another study (Correia et al. 2004) reveal the presence of metallothionein-like proteins in *Gammarus*.

In embryos, Cd has no significant influence on the ecdysteroids levels. Following proposals may explain this observation (i) Embryos have already a stock of (maternal) ecdysteroids which will not be influenced by Cd (ii) Embryos have a defense mechanism so that to oppose to the ecdysteroids toxicity.

Therefore, abnormalities in embryos observed by Geffard et al. (2010) may be rather related to the fact that (i) Cd directly interferes with the actions of ecdysteroids (ii) Cd affect other hormones or mechanism that regulate the embryonic development.

Conclusion

The ecdysteroid system is used by crustaceans and other arthropods as the major endocrine signaling molecules, regulating processes such as molting and embryonic development. In this sense, the present article reports the first data on the characteristics of the ecdysteroids levels during molting of females and in the stage of embryonic development in *G. fossarum*. Our findings develop also, for the first time an in vitro toxicity test in *G. fossarum* for evaluating the effect of Cd on the molting gland, by measuring ecdysteroids production. Results show that cadmium exposure influences the amounts of ecdysteroids produced by Y-organ and therefore the levels of the whole body. Results lead us to (i) propose an influence, by cadmium, *in G. fossarum*, on the mechanism responsible for the production of the ecdysteroids needed for molting (ii) prove that the in vitro toxicity test could present valuable information on endocrine disrupting chemicals in *G. fossarum*.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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