INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 24, No. 3, p. 93-103, 2024

RESEARCH PAPER OPEN ACCESS

Steroid hormone in gonad maturation in the shrimp *Atya scabra* (Leach, 1815) (Crustacea: Decapoda, Atyidae) in the Bia River, Côte d'Ivoire

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Key words: Plantain, Functional parameters, Storage, Polyethylene

http://dx.doi.org/10.12692/ijb/24.3.93-103 Article published on March 07, 2024

Abstract

The objective of this study was to describe gonad development in a population of the freshwater shrimp *Atya scabra* (Leach, 1815) from Bia river, Côte d'Ivoire. Moreover, the levels of the sex hormones P4, T and E2 were measured in the gonads to verify whether they are present in this shrimp, and, if present, also to see if their levels varied according to the gonad development stage. The histological examination of gonads and the quantification of these hormones were done in shrimps collected monthly for 1 year. This study shows the presence of the sex steroid hormones in *A. scabra* for the first time and indicates a relationship of these with the gonad development. The current data provide valuable information on shrimp reproduction, which are necessary for developing sustainable management strategies.

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Introduction

Decapod crustaceans, particularly caridean shrimps, are important components of tropical and subtropical freshwater ecosystems (De Grave *et al.*, 2008; Yeo *et al.*, 2008). Atyidae is the richest caridean family in freshwaters with 359 species/subspecies (De Grave *et al.*, 2008). *Atya scabra* (Leach, 1815) is an atyid with amphi-Atlantic distribution, occurring from Mexico to Brazil, as well as across most Caribbean islands. In Africa, *A. scabra* occurs from Liberia southwards to Angola, as well as the Cape Verde Islands and in the islands in the Gulf of Guinea (Hobbs and Hart, 1982; Melo, 2003; De Grave *et al.*, 2013). This species is of economic importance in Mexico, Venezuela, North eastern Brazil (Holthuis, 1980; Martínez-Mayén and Román-Contreras, 2000) and in Côte d'Ivoire (Kadjo *et al.*, 2016a). Despite its economic importance, studies on the reproduction of *A. scabra* are scarce. Previous studies addressed factors such as breeding season, fecundity, and size at sexual maturity (Almeida *et al.*, 2010; Herrera-Correal *et al.*, 2013; Kadjo *et al.*, 2016b). Gonad development is well-known as a period during which gonads undergo dynamic processes including cell modifications, development of gametes and spawning (Grassé, 1994; Etchian *et al.*, 2004; Sokolowicz *et al.*, 2006). The histological analysis of gonads is a good tool to describe the reproductive activity in crustaceans (Lldora *et al.*, 2000; Sampaio *et al.*, 2011), however, it has not been performed in *A. scabra* up today. In fact, recognizing the seasonal patterns of gametogenesis in commercially important invertebrates is essential for developing management strategies, aimed at protecting spawning stocks (protecting spawning stocks and determining the timing of larval settlement) (Gribben, 2005). Steroid molecules have been detected in many invertebrate phyla and some of them have established hormonal roles (Lafont and Mathieu, 2007). In the phylum Mollusca, sex steroid hormones such as progesterone (P4), testosterone (T) and 17β- estradiol (E2) are considered as main regulators of reproduction (Siah *et al.*, 2002; Gauthier-Clerc *et al.*, 2006). Such studies are very limited in arthropods and very scarce in crustaceans (Paolucci *et al.*, 2002; Coccia *et al.*, 2010; Li *et al.*, 2015; Sujathamma and Dayakar, 2015). More literature on steroids in crustacean species has been published, particularly concerning the detection of either steroid levels or steroid receptors in crustacean tissues, as well as the administration of sexual steroid to enhance reproduction. In this study, we described the stages of gonadal development in a population of *A. scabra* from Bia River, Côte d'Ivoire. Moreover, the gonads of *A. scabra* were investigated for the presence of P4, T and E2 hormones, whose levels were quantified in order to correlate them with gonad development.

Materials and methods

Sampling area

Located in the South-East of Côte d'Ivoire (fig. 1), the Bia River (Vanden Bossche and Bernacsek, 1990) belongs to the Western Guinean ichthyo-region, sector Eburneo-Ghanaian (Konan *et al.*, 2013). This tropical river has 300 km in length (05°30'-05°50'N and 03°-03°15'W), encompasses an area of 9,300 km² and has a mean annual flow of 83 m^3s^{-1}. In addition, two dams (Ayamé 1 and Ayamé 2) were built on this river in 1959 and 1965, respectively (Kadjo *et al.*, 2016a).

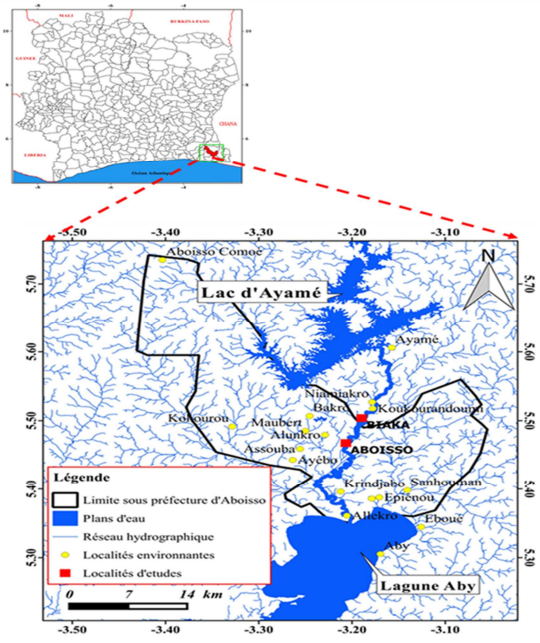


Fig. 1. Map of Côte d'Ivoire showing the Bia river basin and the location of the sampling sites (adapted from Kadjo *et al.*, 2016b).

Samplings were conducted monthly for 1 year in the main course of the Bia river, specifically at Biaka and Aboisso (Aboisso department, Sud Comoé region), both located downstream of the Ayamé 1 and Ayame 2 dams. Aboisso is an urban zone; whereas Biaka is a rural area. We sampled shrimps in four stations: T1 (5°27'N 3°12'W) and T2 (5°28'N 3°12'W) in Aboisso and T3 (5°28'N 3°11'W) and T4 (5°30'N 3°11'W) in Biaka (Fig. 1). These stations have wet equatorial climate, with a high dry season (December to April), a high rainy season (May to July), a low dry season (August to September) and a low rainy season (October to November).

Shrimp collection

The shrimps (30 individuals per month) were collected by hand and by diving (Kadjo *et al.*, 2016a) in rocky bottoms areas of the sampling stations. In each site, animals were rapidly placed in coolers filled with ice and transported to laboratory.

In the laboratory, shrimps were sexed based on the presence or absence of the *appendix masculina* on the 2nd pair of pleopods (Galvão and Bueno, 2000; Almeida *et al.*, 2010). Then, the gonads were removed for histological analysis, as well as for hormone identification and quantification.

Histology of gonads

We performed a histological analysis on the gonads to determine the temporal patterns of the gametogenic development in both females and males, using the method of Martoja and Martoja (1967). Briefly, the gonads were preserved in Bouin's fixative for 24 hours, dehydrated using different ethanol concentrations and embedded in paraffin. Samples were sectioned using a microtome (5 µm thickness) and stained with hematoxylin and eosin for routine light microscopic examination. The data of the sexual maturation of the gonads are illustrated by the Table 1 (Kadjo *et al.*, 2018).

Table 1. Morphological criteria used for the determination of different stages of sexual maturation in *A. scabra*

Stages of gonad development	Females	Males
Undifferentiated	Germinative zone with gonies separated by mesodermal cells (Fig. 3A (1)).	Presence of some diffuse spermatogonies in the mesodermal cells (Figure 3B (1))
Developing	Presence of large gonies in the mesodermal cells (Fig. 3A (2)).	Proliferation of spermatocytes in mesodermal cells (Figure 3 B (2)).
Ripe	Oocyte II appears in the lumen and is isolated from the ovarian acini (Fig. 3A (3)).	Spermatozoa with several flagella distributed in the seminiferous tube (Figure 3B (3)).
Spawning	Mature oocyte II expelled from the follicular cells (Fig. 3A (4)).	Presence of some spermatozoa in the seminiferous tube (Figure 3B (4)).
Spent	The follicular cells retract to form a thin, flaccid layer (Fig. 3A (5)).	Spermatozoid evolved seminiferous tubes from a thin, flaccid layer (Figure 3B (5)).

Kadjo *et al.*, 2018

Hormones identification

Frozen gonadal tissues were thawed and 0.3 g of each gonad was homogenized in 1 mL of ethanol 98%. The homogenate was then centrifuged for 30 min at 3500 rpm. The organic (steroid-containing) phase was then added to 3 mL of buffer solution and 2 mL of this solution were used for hormone quantification. The assay principle combines the two-step competition immune enzymatic method with final fluorescence detection (ELFA). The test steps consist of a succession of suction/discharge cycles of the reaction medium. The strip contains anti-E2 monoclonal antibody (estradiol) or anti-P4 monoclonal antibody (progesterone) or anti-T monoclonal antibody (testosterone) conjugated to alkaline phosphatase and all other reagents. Washing steps then remove the unbound conjugate. During the final revealing step, the substrate (4-methyl-ombelliferil phosphate) is sucked and then pushed back into the cone; the conjugate enzyme catalyzes the hydrolysis reaction of this substrate into a product (4-methyl ombelliferone) whose emitted fluorescence is measured at 450 nm. The conjugate reacts with E2 SPR (estradiol) or P4 SPR (progesterone) or T SPR (testosterone) to give a

fluorescent signal in the presence of the substrate. The value of the fluorescence signal is inversely proportional to the E2 (estradiol) or P4 (progesterone) or T (testosterone) concentration of the sample. At the end of the test, the results are calculated automatically by the instrument in relation to a memorized calibration curve, and then printed. As for the cartridge, it serves as a support for the liquid phase composed of reagents for the immunological reaction ready for use. The levels of the steroid hormones progesterone (P4), testosterone (T) and estradiol-17β (E2) were determined in gonads using the Mini Vidas ® enzymatic method-fluorescent ELFA (Enzyme Linked Fluorescent Assay) (bioMérieux, S.A., France) (Ekins, 1990; Anckaert *et al.*, 2002; Goldsby *et al.*, 2003) according to the manufacturer's recommendations. The detection limits were 0.1 ng mL^{-1}, 0.1 ng mL^{-1} and 9.0 $pgmL^{-1}$ for P4, T and E2, respectively.

Statistical analyses

Data were tested to assess the normality of distribution using a Kolmogorov-Smirnov and the homogeneity of variances checked with the Hartley test. When ANOVA revealed a significant effect ($P \leq 0.05$), a multiple comparison of means was performed using the Tukey test. Data were processed using SAS and graphics were made in SigmaPlot 13.0 for Windows.

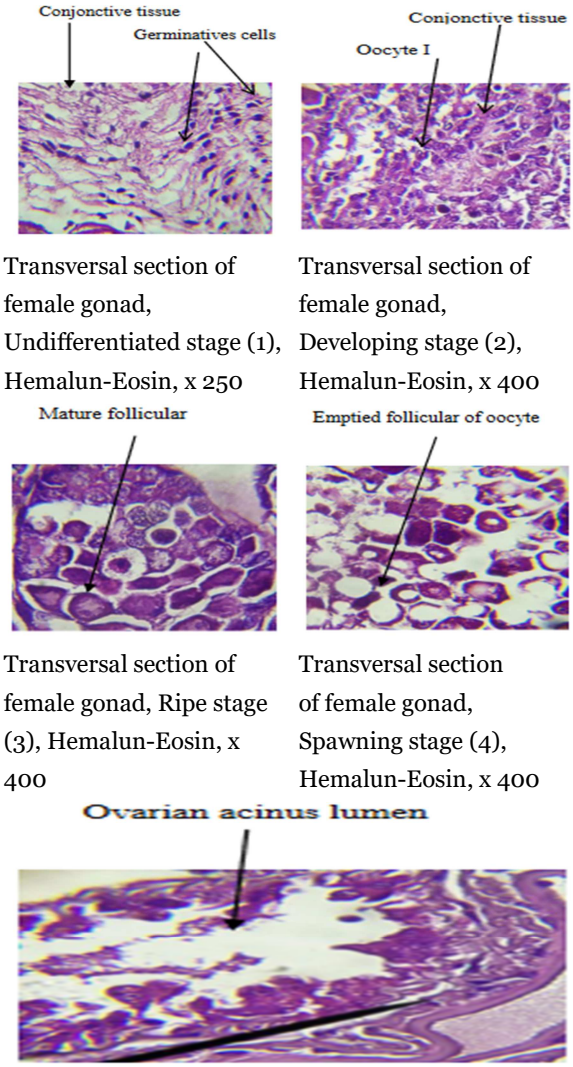
Results

Gonadal development

Gonads were classified into five maturity stages (1) undifferentiated, (2) developing, (3) ripe, (4) spawning and (5) spent (Fig. 2; 3). These stages were determined based on the development and the arrangement of oocytes and spermatogenic lineage cells (Amanat and Qureshi, 2011; Olele *et al.*, 2012).

Hormone assays

We detected P4, T and E2 hormones in the gonads of *A. scabra* and their concentrations varied significantly ($P < 0.05$) in both sexes during gonad development (Fig. 6), as detailing below. Hormone levels are expressed in ng g^{-1} (P4 and T) and pg g^{-1} (E2).



Transversal section of female gonad, Undifferentiated stage (1), Hemalun-Eosin, x 250

Transversal section of female gonad, Developing stage (2), Hemalun-Eosin, x 400

Transversal section of female gonad, Ripe stage (3), Hemalun-Eosin, x 400

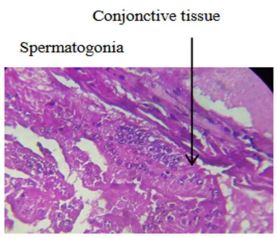
Transversal section of female gonad, Spawning stage (4), Hemalun-Eosin, x 400

Transversal section of female gonad, Spent stage (5), Hemalun-Eosin, x 40

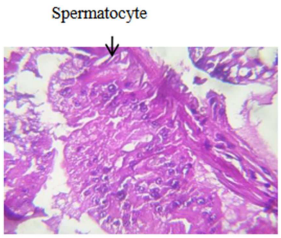
Fig. 2. Structure of female gonadal tissue. Undifferentiated stage (1), developing stage (2), Ripe stage (3), Spawning stage (4), Spent stage (5).

Progesterone (P4) levels

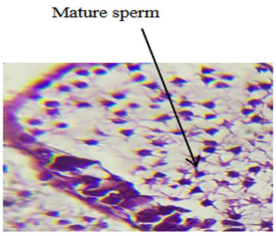
We detected P4 (Fig. 4) in the gonads of all development stages, at both sites studied. In Aboisso, P4 levels in female gonads varied from 1100 ± 150 (in undifferentiated gonads) to 2730 ± 300 (in spawning gonads). Similarly, the concentrations of P4 in females of Biaka were low (1210 ±118) in undifferentiated gonads and high (3480 ± 120) in spawning gonads. Females from both sites showed a similar pattern of P4 levels throughout gonadal development: there was a high increase from the developing to the ripe stage, reaching a peak in the spawning stage and finally decreasing in the spent stage.



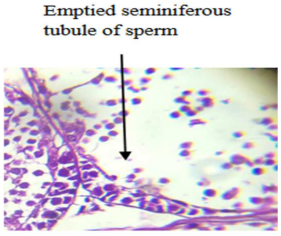
Transversal section of male gonad, Undifferentiated stage(1), Hemalun-Eosin, x 250



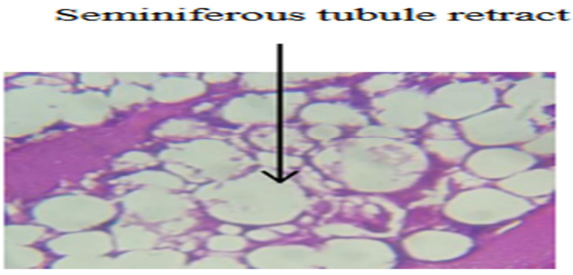
Transversal section of male gonad, developing stage (2), Hemalun-Eosin, x 250



Transversal section of Male gonad, ripe stage (3), hemalun-Eosin, x 400



Transversal section of male gonad, Spawning stage (4), Hemalun-Eosin, x 400



Transversal section of male gonad, Spent stage (5), Hemalun-Eosin, x 400

Fig. 3. Structure of male gonadal tissue. Undifferentiated stage (1), developing stage (2), Ripe stage (3), Spawning stage (4), Spent stage (5).

Moreover, females from Biaka had higher P4 levels during the ripe and spawning stages compared to those from Aboisso. In males, patterns of P4 levels throughout the gonadal development were similar in the two sites. Indeed, there was a significant increase in P4 levels from the developing stage to the ripe stage (where the highest concentrations were observed), and they remained relatively unchanged in the subsequent stages. In Aboisso individuals, male P4 levels were 1050 ±121 in undifferentiated stage and 1240 ±240 in developing stages, increasing to 2890±170 in the ripe gonads and remained unchanged along the other stages. In male gonads of shrimps from Biaka, P4 levels were 980 ± 60 in undifferentiated gonads and 1087 ± 90 in developing gonads, increased to 2730 ± 120 in the ripe stage and to remain quite stable with values of 2500 ± 170 for spawning stage and 2480 ± 222 for spent stage.

Testosterone (T) levels

Testosterone levels in females from Aboisso varied from 9.12 ± 0.7 to 24 ± 5.37. The minimum level was observed at the beginning of the gametogenesis (undifferentiated stage), and the maximum value was recorded in the ripe stage (Fig. 5). Testosterone level gradually decreased during the rest of gametogenesis being 17.41 ± 3.48 at the spent stage. Concerning the females from Biaka, T levels decrease from the undifferentiated stage to the developing stage, which presented the minimum T value (7.56 ± 0.15). Then, T levels gradually increased and the maximum value (21 ± 1.85) was observed in spawning stage. Finally, in the last maturity stage (spent) a slightly lower testosterone level (17.41 ± 3.48) was recorded. We also verified that Biaka females had higher T levels in the undifferentiated stage than those from Aboisso, while lower levels were observed for the developing and ripe stages (Fig. 5). Figure 5 shows the seasonal frequency of the different stages of gonad maturation at each site. Our results demonstrated that the female and male shrimps with spawned and spent gonads were present in high proportions in all seasons.

In males from the both sites, testosterone patterns levels were similar throughout the gonadal development; no significant differences ($P > 0.05$) were detected (Fig. 5). Testosterone levels varied from 1.35 ± 0.19 to 13.28 ± 1.32 in males from Aboisso and from 1.94 ± 0.27 to 12 ± 0.44 in males from Biaka. In general, T levels gradually increased from the undifferentiated stage to the ripe stage. We recorded a marked decrease in T levels during the spawning stage, when the minimum T level was recorded. Finally, there was a marked increase in T levels at the spent stage, when the maximum level was recorded.

Estradiol-17β (E2) levels

We did not detect E2 in undifferentiated gonads in never sexes (Fig. 6).

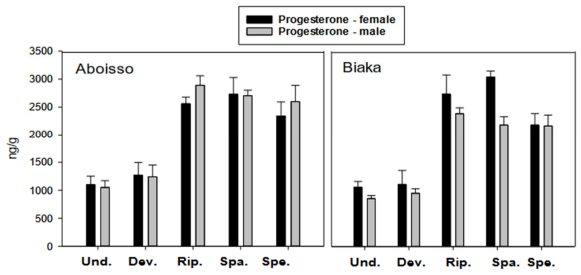


Fig. 4. *Atya scabra* (Leach, 1815). Levels of Progesterone (P4) in the gonads during gonadal maturation in females and males collected at two locations (Aboisso and Biaka) in Bia River, Côte d'Ivoire. Each value indicates the mean ± S.D. of duplicate measurements

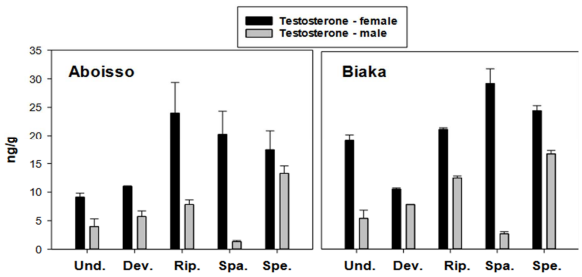


Fig. 5. *Atya scabra* (Leach, 1815). Levels of Testosterone (T) in the gonads during gonadal maturation in females and males collected at two locations (Aboisso and Biaka) in Bia River, Côte d'Ivoire. Each value indicates the mean ± S.D. of duplicate measurements

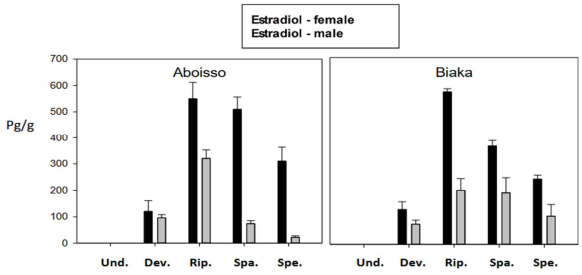


Fig. 6. *Atya scabra* (Leach, 1815). Levels of Estradiol-17β (E2) in the gonads during gonadal maturation in females and males collected at two locations (Aboisso and Biaka) in Bia River, Côte d'Ivoire. Each value indicates the mean ± S.D. of duplicate measurements

The lowest detectable levels of E2 in females from Aboisso and Biaka were, respectively, 120 ± 41 and 148.58 ± 33 which were found indeveloping gonads. The E2 levels peaked during the ripe stage reaching 550 ± 62in females from Aboisso and 654 ± 13.33 in females from Biaka. Then, E2 levels gradually decreased until the spent stage, reaching a concentration of 310 ± 54 from Aboisso and 277.76 ± 17.26 from Biaka. When compared to Aboisso, E2 levels of females from Biaka were significantly ($P < 0.05$) higher for ripe gonads, and lower for spawning and spent gonads. In males (Fig. 8), the maximum E2 concentrations of 321 ± 32 from Aboisso and 230 ± 50 from Biaka were recorded in developing gonads. The lowest levels of E2 in males were observed in spent gonads being 22 ± 6 from Aboisso males and 120 ± 50 from Biaka males.

Discussion

In caridean shrimps, the occurrence of both sexes with spawning and spent gonads during the sampling period are strong indications of a continuous reproduction (Mossolin and Bueno, 2002; Bauer, 2004; Soomro *et al.*, 2011). Accordingly, a recent study focusing on the fertility in *Atya scabra* in the Bia river conducted, reported the constant appearance of ovigerous females throughout the year (Kadjo *et al.*, 2016b).

In *A. scabra,* a new ovarian maturation frequently occurred during egg incubation and a new spawning occurred followed soon after larval hatching (Galvão and Bueno, 2000; Almeida *et al.*,2010). Almeida *et al.* (2010) stated: "Dissection of females carrying eggs in the final incubation stage or larval-release stage, showed ovaries in advanced vitellogenesis, indicating that these females continuously produce broods and therefore are soon ready for a new clutch". We believe that the continuous breeding activity is permitted by the very low fluctuations of environmental factors in tropical aquatic ecosystems as opposed to those encountered by invertebrates in temperate waters. Hormis, les paramètres environnementaux et l'alimentation, l'ablation bilateral eyestalk induit un développement ovarien in the non- reproductive period in female kuruma prawn *Marsupenaeus japonicus* (Okumura and Sakiyama, 2004). Despite, parameters environmental and food availability, bilateral eyestalk ablation induced ovarian development in the non-reproductive period in

female kuruma prawn *Marsupenaeus japonicus* (Okumura and Sakiyama, 2004).

Our research evidenced the occurrence of some vertebrate-like sex steroid hormones (VLSHs): progesterone (P4), testosterone (T) and estradiol-17β (E2) in the gonads of *A. scabra*. The detection of P4, E2 and T in both sexes are expected since several studies have highlighted the VLSHs in crustaceans (Cai *et al.*, 2001; Lafont and Mathieu, 2007; Nagaraju, 2011; Swetha *et al.*, 2011; Mirheydari *et al.*, 2014; Merlin *et al.*, 2016). For example, Cai *et al.* (2001) identified P4 and E2 in the ovaries of the shrimp *Penaeus chinensis* (Osbeck, 1765) and Burns *et al.* (1984) identified T in the testes of the American lobster *Homarus americanus* H. Milne Edwards, 1837. Okumura and Sakiyama (2004) detected vertebrate-type steroid hormones (estradiol-17β, estriol, progesterone, testosterone, and 11-ketotestosterone) in female kuruma prawn *Marsupenaeus japonicus* in hemolymph and ovaries. Estradiol and progesterone were identified in hemolymph, hepatopancreas and ovaries in fully mature shrimps, *Penaeus monodon*, only by Quinitio *et al.*, 1994.

P4 levels in *A. scabra* were high compared the other hormones and E2 levels were far higher than T concentrations. However, in female shrimp, *Penaeus monodon*, a peak in estradiol level was noted in mature shrimps (Stage 5). The progesterone levels in the hemolymph were high in shrimps with mature ovaries (Stages 4 and 5) while those with immature ovaries (Stages 2, 3 and spent) were low or undetectable (Quinitio *et al.*, 1994).

Some studies showed that the gonads of the crustaceans are capable of synthesising sex steroid hormones (Lafont and Mathieu, 2007). For example, some key enzymes for steroid hormone biosynthesis (i.e., steroidogenesis) such as C17-C20 lyase, 17α-hydroxylase, 17β-hydroxysteroid dehydrogenase (17β-HSD) and aromatase were detected in the ovary of the shrimp *Marsupenaeus japonicus* (Spence Bate, 1888) (Summavielle *et al.*, 2003). On the contrary, other results are consistent with the hypothesis that the hepatopancreas may be the primary site of steroid synthesis and that hormones are then transported to the developing ovaries (Wang *et al.*, 2014; Merlin *et al.*, 2016). In addition, the presence of receptors of sex steroids as P4 and E2 has been demonstrated in gonads and hepatopancreas of crustaceans (Paolucci *et al.*, 2002). Despite of the results reported by Summavielle *et al.* (2003), no aromatase activity has been found by Swevers *et al.* (1991), in neither the hepatopancreas nor gonads of three crustacean species (*Cancer pagurus*, *Macrobrachium rosenbergii* and *Artemia salina*), by using radiolabeled precursors.

The concentrations of sex hormones measured here varied significantly during the gametogenesis, thus suggesting a possible role of these hormones in the regulation of the oogenesis and spermatogenesis regulation as reported in other crustaceans.

The female shrimps from Biaka had high steroid hormone levels in the ripe and spawning stages as compared to females from Aboisso (Fig. 5A). One explanation is the presence of females with ovaries in an advanced stage of development (ripe and spawning stages). Another possible explanation is that Biaka is a rural area and less polluted than the urban area of Aboisso. Indeed, it is well documented that some environmental pollutants namely endocrine-disrupting compounds (EDCs) present in the aquatic ecosystems (Rahman *et al.*, 2009) might interfere with the steroid hormone metabolism in wildlife (Pellerin *et al.*, 2004; Rodríguez *et al.*, 2007). In *Penaeus monodon*, the *in vitro* incubation of prawn previtellogenic oocystes with P4 and 17α-hydroxyprogesterone (17α-OHP4) extracted from polychaetes, significantly increased percentages of vitellogenic oocystes and oocyste with cortical rod (Meunopol *et al.*, 2007). Theses authors showed that P4 acted in the final maturation oocystes while 17 α-OHP4 had more effects on vitellogenic oocystes. In the same prawn, receptors named progesterone receptor-related protein p23 (Pm-p23), was isolated from EST analysis of the cDNA library established from vitellogenic ovaries (Preechaphol *et al.*, 2010).

The E2 level was too low to be detected in undifferentiated gonads of females and male. The maximum E2 level of 654±13.33 pgg^{-1} was found in female shrimps (it was 321±32 pgg^{-1} in males) suggesting that E2 is involved in oogenesis as a vitellogenesis stimulating ovarian hormone (VSOH) as demonstrated in crustaceans (Huberman, 2000; Summavielle *et al.*, 2003; Coccia *et al.*, 2010). Atrazine is known as an potential endocrine disruptor for freshwater species. But in the female *Procambarus clarkii*, exposure to atrazine increase the level of estradiol levels in the ovary and hepatopancreas. This is not the case with vitellogenin. No effect was observed on lipid peroxidation (Silveyra *et al.*, 2018). As far as T is concerned, its fluctuating levels in both males and females suggest that this steroid plays a role in the regulation of the reproduction in *A. scabra*. Mirheydari *et al.* (2014) observed changes in T levels during testes maturation in male crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823), suggesting a role in the regulation of late spermatogenesis and spawning. It is well documented that the regulation of the reproduction in crustaceans is controlled by the interaction of environmental factors (like temperature, pH, salinity and food availability) and internal factors (i.e., hormones) (Swetha *et al.*, 2011).

Conclusion

In conclusion, our results show that *A. scabra* has a higher reproductive activity during the two rainy seasons and they confirmed a continuous-seasonal reproductive pattern which was observed in other tropical populations of this species. The continuous-seasonal reproduction is permitted by the very low variations in environmental factors in the Bia river. Indeed, this pattern contrasts to what is observed in temperate species which display opportunistic tactics. Thereby, our study provides valuable information on gonad development which is necessary for developing sustainable management strategies. As far as we know, this study is the first report of sex steroid hormones in *A. Scabra* and the first to show a relation between sex hormones and gonad development in this species. Further studies are needed to evaluate the effects of environmental factors on the steroidogenesis and vitellogenesis in this Atyid shrimp.

Acknowledgements

The authors are sincerely grateful to the Oceanologic Research Center (Abidjan) for providing materials and support to conduct this work. We also would like to thank Mr. DJA Assi Patrice for help in the collection of shrimps and Mr. KONE Kanakounou Jean-Marie for his technical assistance in this research. AOA thanks Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for a Research Scholarship support (305939/2015-7).

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