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Occurrence of Vertebrate-Like Steroids in the Male Narrow-Clawed Crayfish *Astacus leptodactylus* (Eschscholtz, 1823) from Iran during the Annual Reproductive Cycle

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Abstract: In this study, the vertebrate-like steroids, testosterone, 17β-estradiol and progesterone were measured in the hemolymph of the male crayfish *Astacus leptodactylus* from Iran throughout the reproductive cycle. Gonadosomatic Index (GSI) of the *Astacus leptodactylus* male was low in June and August while increased significantly in November and January. The high levels of testosterone registered in November and January go along with the testis maturation, suggesting a possible role of testosterone in the regulation of late spermatogenesis and spawning. 17β-estradiol fluctuations were compatible with its role in reproduction. Indeed, 17β-estradiol was undetectable in June, after that it started to gradually increase to reach the highest level in January. Progesterone fluctuations were apparently unrelated to the reproductive cycle, although its role as precursor of active metabolites cannot be ruled out. Taken together the data presented here point at a remarkable role for sex steroids on reproductive biology of *Astacus leptodactylus*, although further studies are needed to clarify the mechanisms regulating metabolism, synthesis and activity of these fundamental hormones and to understand their detailed biological roles.

Key words: *Astacus leptodactylus* · GSI · Testosterone · 17*β*-Estradiol · Progesterone

INTRODUCTION

It is well known that many aspects of vertebrate reproduction are under the control of sex steroids and a great deal of evidence has been accumulating, showing that it may also be the case of crustaceans [1, 2].

Sex steroids have been shown to be synthesized in the gonads of crustaceans, along with the enzymatic capacity to synthesize them [3].

A positive relationship between vitellogenin, a phospholipoglicoprotein synthesized by the hepatopancreas and accumulated into the oocytes, in the hemolymph and circulatory levels of both progesterone and 17β-estradiol have been observed for shrimp *Paenaeus monodon* [4], prawns [5] and crabs [6]. Fluctuating levels of 17β-estradiol and progesterone in the ovary and hemolymph at different vitellogenic stages of the crab *Scylla serrata* were also reported [7]. Moreover, the stimulatory effects of 17β-estradiol and progesterone on ovarian growth in decapod crustaceans have been reported [8-10]. Injection of progesterone induced ovarian development in the shrimp *P. hardwickii* [11]. Progesterone and 17β-estradiol apparently stimulated vitellogenin gene expression in both hepatopancreas and ovary explants of *Metapenaeus ensis* [12]. Administration of 17α-hydroxyprogesterone stimulated ovarian growth

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and vitellogenesis in the kuruma prawn *Metapenaeus japonicus* [13]. 17β-estradiol stimulated vitellogenesis by ovary fragments *in vitro* [5] and *in vivo* in crayfish [9]. Injection of 17α-hydroxyprogesterone induced ovarian maturation in the crab *Oziotelphusa senex senex* [14].

The possibility that vertebrate-like sex steroids may play a regulatory role in crustacean reproduction is reinforced by the presence of specific receptors within the cell, necessary to carry on the steroid regulatory functions [15, 16]. So far, immunological evidence for progesterone receptor in the ovary and both progesterone and 17β-estradiol receptor in the hepatopancreas of the crayfish *Austropotamobius pallipes* [17] and estrogen and androgen receptors in the brain and the thoracic ganglion mass of the mud crab *Scylla paramamosain* [18] have been reported, suggesting a feedback mechanism recalling the hypothalamus-pituitary-gonads axis operating in vertebrates [19].

The knowledge of the regulation of crustacean reproduction is certainly relevant for both the crustacean industry and environmental conservation strategies. Thus, it is surprising that there are virtually no studies on vertebrate-type steroids in male decapods crustaceans. Some studies suggest that most androgen metabolites identified in invertebrates are common in vertebrates and sex differences exist, with males showing a higher degree of testosterone and its active form dihydrotestosterone than females [20-22].

In this study, the fluctuation pattern of testosterone, 17β-estradiol, progesterone in the hemolymph of the male crayfish *Astucus leptodactylus* was determined during the reproductive cycle. Sex steroid level fluctuations were discussed in light of the morphological modifications of testis.

MATERIALS AND MEYHODS

Animals: Sixty male crayfish were collected by local fishermen from Aras Dam Lake, Western-Azerbaijan, Iran in June, August and November 2011 and January 2012. Total body length was measured to the nearest-.1 mm with a caliper, from the rostral apex to the posterior median edge of the telson and ranged between 79.8 and 172.2.

The carapax length ranged between 47.4 and 67.8 mm. The wet weight was measured to the nearest-.1 g and ranged between 58.7 and 99.6 g. Soon after the catchment, crayfish were transferred to the laboratory and anaesthetized on ice. Hemolymph was withdrawn by using 2 ml syringe with 9 mmol EDTA with pH 7 as an anticoagulant solution for crayfish [23] and centrifuged at 800xg for 15 min at 4°C. The supernatant was collected and stored at -80°C until further use.

Gonadosomatic Index (GSI): Crayfish were dissected and the testes removed and weighted using a digital balance. Testes were treated for histological analysis. The gonadosomatic index (GSI) was calculated as follows: wet weight of testes/ total body weight X 100.

Steroid Measurement: Testosterone was measured by ELISA method, using IBL kit (IBL International GmbH, Germany) according to the manufacturer directions. The antibody had 100% cross reactivity for Testosterone (8.67% for 11β-OH-Testosterone and 3.24% for 11-α-OH-Testosterone) and the minimum detectable concentration was-.07 ng/ml. 17β-estradiol was measured by ELISA method, using IBL kit (IBL International GmbH, Germany) according to the manufacturer directions. The antibody had 100% cross reactivity with 17β-estradiol and the minimum detectable concentration was 9.7 pg/ml. Progesterone was measured by ELISA method, using dbc kit (Diagnostics Biochem Canada Inc.) according to the manufacturer directions. The antibody had 100% cross reactivity with 11α-OH-Progesterone and the minimum detectable concentration was-.1 ng/ml.

Statistical Analysis: Values were expressed as mean±standard error (SE). Data were analyzed by one-way analysis of variance (ANOVA) and any significant difference was determined at the-.05 level by Duncan's multiple range test. The analyses were carried out with the Statistica version 7.0 statistical package (Statsoft Inc., Tulsa, OK, USA).

RESULTS

GSI: The *Astacus leptodactylus* male GSI ranged from-.50 to 1.21 % throughout the year. The GSI value was low in June (0.50 %) and August (0.52 %), while increased significantly in November (1.21 %) and January (1.12 %) (Fig. 1).

Steroid Fluctuations: Testosterone levels measured in the hemolymph of males of *Astacus leptodactylus* ranged between-.25 and 1.52 ng/ml. The highest level was attained in November and the minimum in June (Fig. 2A).

17β-estradiol levels ranged between 33.14 and 1022.00 pg/ml. The lowest concentration of 17β-estradiol was attained in June when it was undetectable, then started to gradually increase and reached the highest level in January (Fig. 2B).

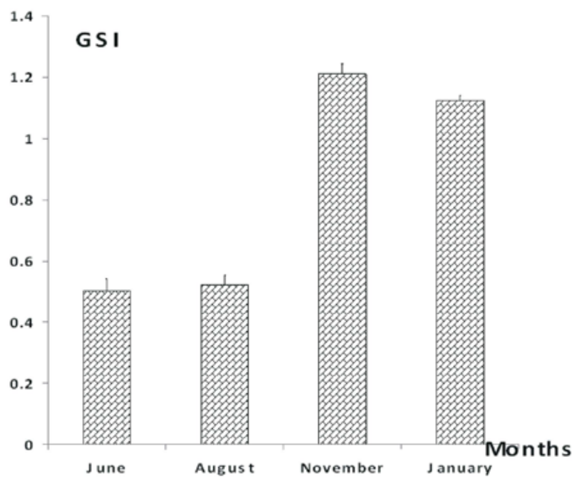

Fig. 1: Gonadosomatic index of the male *Astacus leptodactylus.*

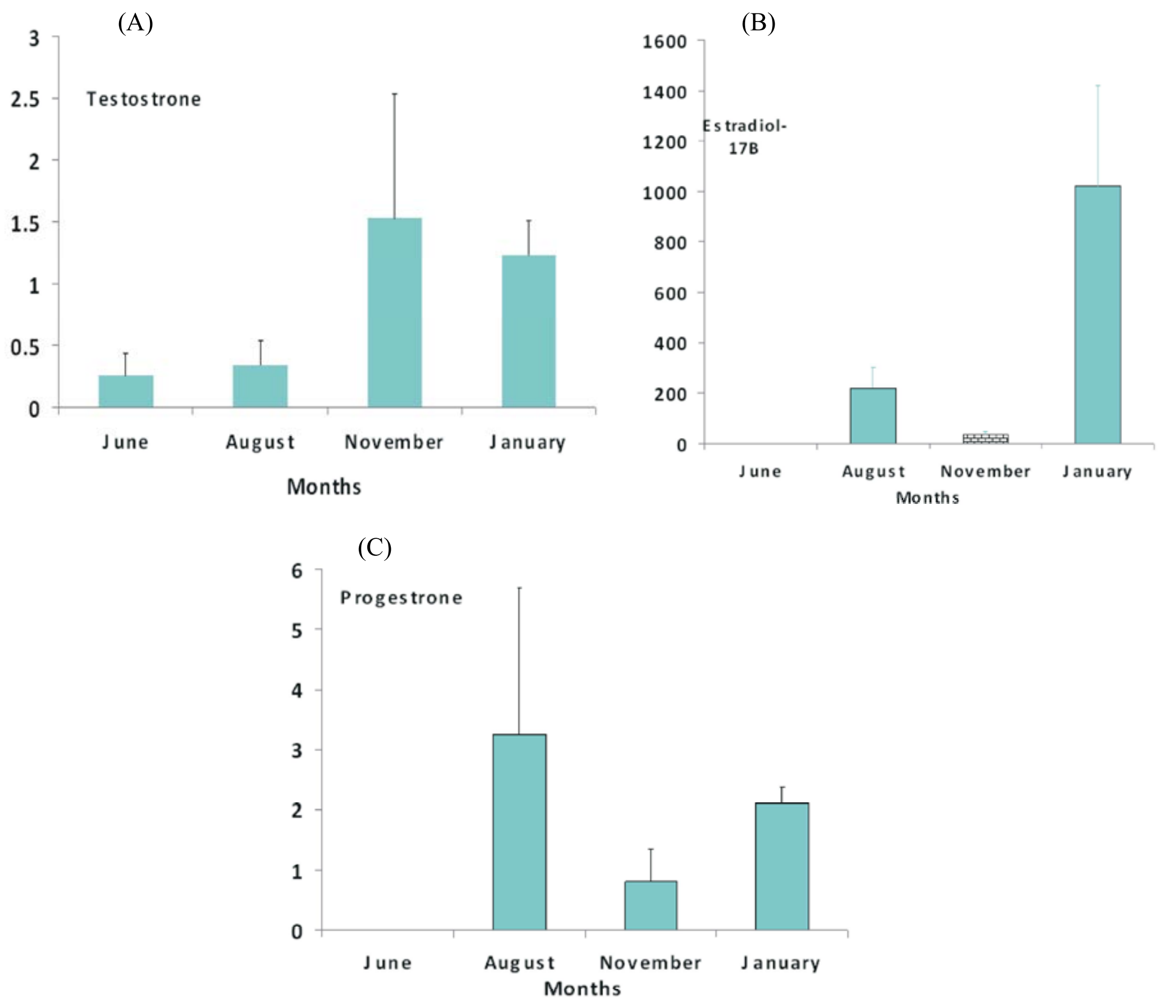

Fig. 2: Hemolymph levels of testosterone (A); 17β-estradiol (B); progesterone (C) in the male *Astacus leptodactylus* throughout the annual reproductive cycle.

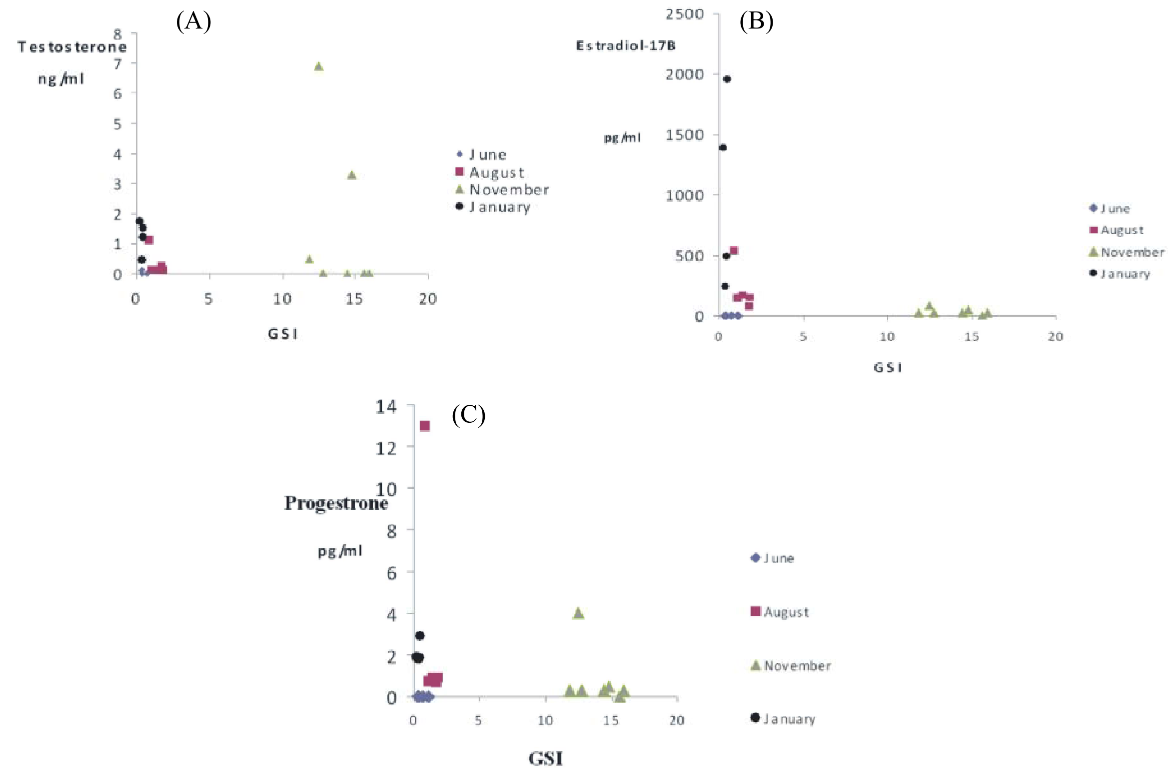

Fig. 3: Linear regression between gonadosomatic index (GSI) and hemolymph levels of testosterone (A), 17β-estradiol (B) and progesterone (C) in the male *Astacus leptodactylus*.

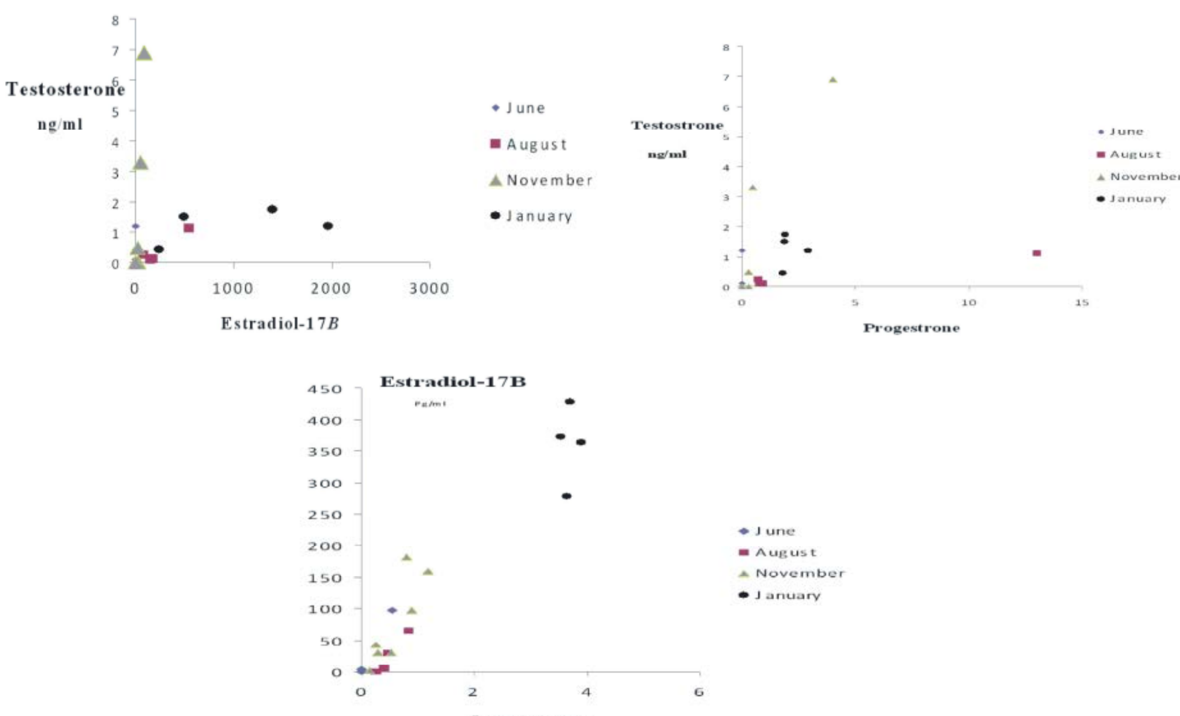

Fig. 4: Linear regression of steroids in the hemolymph of the male *Astacus leptodactylus*. The undetectable values of steroids are given as zero pg/mL (17β-estradiol and progesterone) and ng/ml (testosterone).

Progesterone ranged from-.80 and 2.11 pg/ml. It was undetectable in June and showed the highest level in August (Fig. 2C).

Correlation Analysis: The hemolymph levels of testosterone, 17β-estradiol and progesterone were not significantly correlated to GSI (P>0.05; Fig. 3). The linear regressions didn't show any significant correlation between the studied steroids (P>-.05; Fig. 4).

The undetectable levels of steroids in Figures 2 and 3 are reported as zero pg/ml (17*β*-estradiol and progesterone) and ng/ml (testosterone). Symbols located on the horizontal axis (GSI) indicate steroid undetectable levels.

DISCUSSION

In this study, we report the fluctuations of the sex steroids testosterone, 17β-estradiol and progesterone in the hemplymph of the male crayfish *Astacus leptodactylus* in Iran, throughout the reproductive cycle.

In crayfish the length of the reproductive cycle varies according to the external temperature and the habitat [24]. *Astacus leptodactylus* from Aras Dam Lake, Western-Azerbaijan, mate in autumn, when the temperature starts declining and spawning is complete in January [25, 26]. *Astacus leptodactylus* is a synchronous species with only one or two development stages of spermatogenesis observed in the acini or different regions of the testis [27]. In a recent study carried out by some of the authors, the morphological modifications of the reproductive system in the male of *Astacus leptodactylus* have been described [28]. Although a clear correlation between testosterone levels and the degree of gonad growth, expressed as GSI, cannot be derived in the present study, the fluctuations in testosterone levels are compatible with the reported changes in the testis. The high levels of testosterone registered in November and January (present study) go along with the testis maturation. Indeed, in November the testis shows primary and secondary spermatocytes, while in January spermatids and spermatozoa are dominant [25]. This result suggests a possible role of testosterone in the regulation of late spermatogenesis and spawning.

In vertebrates and rogens are essential for male fertility and the maintenance of spermatogenesis [29, 30]. Testosterone is the androgen in the testis that is responsible for supporting spermatogenesis. In the absence of testosterone or functional androgen receptors, males are infertile because spermatogenesis rarely progresses beyond meiosis [31-33]. Although testosterone has been known to be essential for male fertility in vertebrates [34] the investigation of its involvement in supporting spermatogenesis in invertebrates is only at the beginning. A remarkable role for testosterone on reproductive biology of the male of echinoderms, particularly sea urchins, has been proposed [35]. Moreover, indirect evidence supporting a regulatory role for androgens on gonad maturation and spermatogenesis come from studies on the destructive effect of xenobiotics mimicking steroids [36].

As far as 17β-estradiol is concerned, it is clear that 17β-estradiol concentrations are lower than testosterone levels, possibly reflecting a more important role for this hormone in females. Intriguingly, in female *Astacus leptodactylus* 17β-estradiol levels [25, 26] are lower than in the male (present study). However, it is noteworthy that testosterone and 17β-estradiol are no longer considered male only and female only hormones. Both hormones are important in both sexes. Estrogen receptors are present in the testis, efferent ductless and epididymis of most species [37]. Although estrogen effects in the developing male are important, it has not been proven that estrogen has a role in the adult male reproductive organs [38]. Interesting is the finding that cytochrome P450 aromatase, which is capable of converting androgens into estrogens, is present in the testis [39]. In particular, testicular germ cells and epididymal sperm contain aromatase and synthesize estrogen [40]. It is likely that the high 17β-estradiol levels registered in January are responsible for spermatozoa maturation during this stage of the reproductive cycle of *Astacus leptodactylus*. In general, circulating 17β-estradiol may function as a precursor of testosterone. Unfortunately, information is lacking about the presence of aromatase, the enzyme catalysing the transformation from estrogens to androgens, in the testis of crayfish.

Progesterone fluctuations did not correlate with the GSI in males. A role for progesterone in gonad regulation has been proposed in female crustaceans. In the mole crab *Emerita asiatica* and freshwater prawn *Macrobrachium rosenbergii* the trend in progesterone level in all tissues during different molt and reproductive stages was remarkably similar to that of 17β-estradiol, suggesting that progesterone may have a role in the post-vitellogenic meiotic maturation of the oocytes, as in vertebrates [41]. Although speculative, we can hypothesize that progesterone is a precursor of active metabolites in *Astacus leptodactylus*. Indeed, in the shrimp *Metapenaeus japonicus* the ovary is capable of synthesizing 17β-estradiol from progesterone, evidencing the presence of 17α-hydroxylase, C_{17}-C_{20} lyase, 17β-hydroxysteroid dehydrogenase (17β-HSD) and aromatase.

Enzymatic activities of aromatase, 3β-HSD and 17α-hydroxylase ahave been also detected in the hepatopancreas [42].

In conclusion, if we consider all the data presented here, a remarkable role for sex steroids on reproductive biology of *Astacus leptodactylus*, appeared to be evident. Further studies are needed to clarify the mechanisms regulating metabolism, synthesis and activity of these fundamental hormones and to understand their detailed biological roles.

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