Acta Zoologica
Published for The Royal Swedish Academy of Sciences and The Royal Danish Academy of Sciences and Letters

Acta Zoologica (Stockholm) **94**: 462–470 (October 2013) doi: 10.1111/j.1463-6395.2012.00575.x

Effect of long-term injection of dopamine on the ovarian growth of *Cherax quadricarinatus* juvenile females (Parastacidae, Decapoda)

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Keywords:
Cherax quadricarinatus, dopamine, female juveniles, oocyte atresia, ovarian growth

Accepted for publication:
04 June 2012

Abstract

Tropea, C. and López Greco, L.S. 2013. Effect of long-term injection of dopamine on the ovarian growth of *Cherax quadricarinatus* juvenile females (Parastacidae, Decapoda). —*Acta Zoologica* (Stockholm) **94**: 462–470.

The neurotransmitter dopamine (DA) modulates many physiological processes in decapod crustaceans, including reproduction. This study was aimed at evaluating whether the long-term injection of DA affects ovarian growth in the freshwater crayfish *Cherax quadricarinatus*. Three experiments were performed with females of different mean initial size: 4.15 g (Group A); 9.65 g (Group B); and 13.98 g (Group C). Treated females were injected with DA and control females with physiological saline twice a week for 90 (Group B), 105 (Group A) and 120 (Group C) days. At the end of the experiments, the animals were killed, and the stage of ovarian development, gonadosomatic index, and mean oocyte diameter were determined. DA had a differential effect according to female size: it negatively affected ovarian growth of females in a weight range of 4–14 g and had no effect on ovarian maturation when injected to females with an initial weight of 14 g. The results are compared with previous reports in freshwater decapod crustaceans.

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Introduction

Many studies have addressed the role of neurotransmitters in several physiological functions of decapod crustaceans, with special attention given to serotonin. Both *in vitro* and *in vivo* studies have provided evidence of its stimulatory effect on ovarian maturation in several shrimp species (Alfaro *et al.* 2004; Wongprasert *et al.* 2006, Santhoshi *et al.* 2009), the fiddler crab *Uca pugilator* (Richardson *et al.* 1991), the red swamp crayfish *Procambarus clarkii* (Kulkarni *et al.* 1992; Sarojini *et al.* 1995b), and the freshwater prawn *Machrobrachium rosenbergii* (Meeratana *et al.* 2006).

Dopamine (DA) is a neurotransmitter widely distributed in the nervous system and hemolymph of decapod crustaceans (Elofsson *et al.* 1982; Elekes *et al.* 1988; Barthe *et al.* 1989; Mercier *et al.* 1991; Wood and Derby 1996; Tierney *et al.* 2003; Tinikul *et al.* 2009a). However, studies about the role of DA in ovarian development are scarce, involving the freshwater decapod species *M. rosenbergii*, *P. clarkii*, and *Oziotelphusa senex senex*. These studies demonstrated that DA injection result in depressed vitellogenin synthesis, a decrease in mean oocyte diameter (MOD) and gonadosomatic index (GSI), and an extended ovarian maturation period (Sarojini *et al.* 1995c; Chen *et al.* 2003; Tinikul *et al.* 2008; Tinikul *et al.* 2009b; Sainath and Sreenivasula Reddy 2011). In addition, Tinikul *et al.* (2008) showed that the levels of endogenous DA in the brain, thoracic ganglia, and ovaries of *M. rosenbergii* are highest at ovarian stages I/II (onset of ovarian maturation) and then decline to the lowest values when the ovaries are fully mature (stage IV).

In crustaceans, ovarian growth involves two phases: (i) primary vitellogenesis (or pre-vitellogenesis), which entails the

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synthesis of glycoproteins and lipoproteins in the oocytes called primary (Charniaux-Cotton and Payen 1988); and (ii) secondary vitellogenesis (or vitellogenesis), which entails the synthesis of vitellogenin (precursor of vitellin, the main yolk protein) in the primary oocytes, and/or its incorporation from the hemolymph. The vitellogenin is then transformed into a lipoglycocarotenoprotein and stored as droplets in the cytoplasm (reviewed by Subramoniam 2011). This process demands a high amount of energy and results in a fast increase in the diameter of the oocytes, which are called secondary.

Vitellogenesis is modulated by two antagonistic hormones: the gonad-stimulating hormone (GSH; not characterized at present), which is proposed to be secreted by the brain and thoracic ganglia, and the gonad-inhibiting hormone (GIH), which is produced and secreted in the eyestalks (Sarojini *et al.* 1995a; Fingerman 1997). As DA has not been detected in the eyestalks and either intact or eyestalk-ablated animals responded in the same way to the administration of this neurotransmitter, it was suggested in some of the studies previously mentioned that DA inhibits ovarian maturation by inhibiting the release of GSH rather than by enhancing the release of GIH (Chen *et al.* 2003; Tinikul *et al.* 2009b).

On the other hand, several studies evaluated the effect of spiperone (a DA antagonist) administration on ovarian growth in decapod crustaceans, and showed increased values of GSI and MOD following such procedure (Cahansky *et al.* 2002; Rodríguez *et al.* 2002; Zapata *et al.* 2003; Alfaro *et al.* 2004).

The reproductive biology of females of the freshwater crayfish *Cherax quadricarinatus* has been extensively studied. The species breed in the field up to five times throughout the year (Sammy 1988), except in the subtropical and temperate zones, where there is a recognizable spawning season lasting over 6 months during spring and summer. Females spawn up to three times during the spawning season, when water temperature is above 21 °C, and there are more than 13 h of daylight (Jones 1997). Several studies characterized the physiological and morphological changes associated with ovarian development, during the first reproductive cycle, at first maturation or along ontogeny (Sagi *et al.* 1996; Abdu *et al.* 2000; Serrano-Pinto *et al.* 2003; Rodríguez-González *et al.* 2006; Vazquez *et al.* 2008). Moreover, the endocrine regulation of reproduction has been investigated, and endocrine manipulation of females (e.g. administration of methyl farnesoato, and dopaminergic and enkephalinergic antagonists) has been performed to improve ovarian growth and reproductive performance (Sagi *et al.* 1997; Silkovsky *et al.* 1998; Soroka *et al.* 2000; Abdu *et al.* 2001; Cahansky *et al.* 2011). However, to our knowledge, the effects of long-term administration of DA (through its injection) on the ovarian development of *C. quadricarinatus* have never been reported in the literature.

Based on the considerations mentioned above, the objective of the present study was to evaluate whether the long-term injection of DA affects gonad growth in *C. quadricarinatus* juvenile females.

Materials and Methods

Animals

Juveniles were obtained under laboratory conditions, from a reproductive stock supplied by the Farm Las Golondrinas, Entre Ríos, Argentina. Six ovigerous females (mean wet body weight: 62.62 ± 7.31 g) were placed individually into glass aquaria of 33.5 × 25 × 19 cm containing 8 L of dechlorinated tap water, under continuous aeration (pH 7.5, hardness 80 mg/L as $CaCO_3$ equivalents). Each aquarium was provided with a PVC tube (10 cm in diameter and 25 cm long) as shelter, the water temperature was held constant at 27 ± 1 °C by ATMAN® (Guangdong, China) water heaters (100 W, precision 1 °C), and the photoperiod was 14 L : 10 D, as used in previous studies (Jones 1995, 1997). Females were fed daily *ad libitum* with *Elodea* sp. and commercial food for tropical fish {Tetracolor; TETRA® [Tetra holding (US) Inc., Blacksburg, Germany], approximate composition: min. crude protein 47.5%, min. crude fat 6.5%, max. crude fiber 2.0%, max. moisture 6.0%, min. phosphorus 1.5% and min ascorbic acid 100 mg/kg} (Vazquez *et al.* 2008; Tropea *et al.* 2010). After reaching the free-living stage III (Levi *et al.* 1999), juveniles were separated from their mothers and they were grown under the same laboratory conditions during 90–240 days until the beginning of the experiments.

At the end of the growing period, three groups of females were randomly selected from the pool of juveniles:

Group A: 42 females weighing 4.15 ± 0.02 g

Group B: 36 females weighing 9.65 ± 0.10 g

Group C: 24 females weighing 13.98 ± 0.06 g

According to Vazquez *et al.* (2008), females of approximately 4 g (Group A) have stage II ovaries, which are composed exclusively of primary (or pre-vitellogenic) oocytes with a homogeneous and acidophilic cytoplasm and round basophilic follicular cells surrounding them. Females of approximately 9 g (Group B) have mainly stage II ovaries, with larger primary oocytes than those of females from Group A, and they can also present stage III ovaries, which are composed of primary oocytes (around 50%) and oocytes beginning secondary vitellogenesis (around 50%). Finally, females of approximately 14 g (Group C) have mainly stage III ovaries, with oocytes in a more advanced stage of secondary vitellogenesis. Ovaries at stages II and III are considered to be primary-vitellogenic ovaries (i.e. immature ovaries composed exclusively or mainly of primary oocytes). Ovarian stage IV is the only stage that corresponds to secondary-vitellogenic ovaries (mature ovaries) and occurs in females with a weight >18 g. These ovaries are composed mainly of secondary oocytes (around 73%), which have an eosinophilic cytoplasm containing yolk

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droplets and globules, and are surrounded by flat and pycnotic follicular cells (Abdu *et al.* 2000; Vazquez *et al.* 2008).

Based on these previous observations, an experiment was performed with each group of females (a total number of three experiments), to evaluate the effect of DA administration on the different phases of ovarian growth (primary and secondary vitellogenesis).

Experimental design

For each experiment, females were randomly assigned to one of the following treatments:

Control: injection of crayfish physiological saline (PS) containing 12 g/L NaCl, 0.4 g/L KCl, 1.99 g/L $CaCl_2.H_2O$, 0.31 g/L $MgCl_2.6H_2O$, and 0.2 g/L $NaHCO_3$ (Van Harreveld 1936), in a final volume of 0.05 mL.

Dopamine: injection of DA (Sigma®, Sigma-Aldrich Inc., St. Louis, MO, USA) dissolved in PS, at a dose of 2.5×10^{-6} mol/animal in a final volume of 0.05 mL, according to Chen *et al.* (2003).

Females were injected twice a week at the base of the forth pair of walking legs. The injections were performed alternatively in the right and left pereiopods. Each experimental group was run in triplicate (three aquaria per treatment), with 7, 6, and 4 crayfish per aquarium for Group A, Group B, and Group C, respectively (29, 25, and 17 animals/m^2, respectively).

The glass aquaria ($60 \times 40 \times 30$ cm) contained 30 L of dechlorinated tap water with continuous aeration. PVC tubes (5 cm in diameter and 10 cm long) and an onion bag mesh were used as shelters. The experiments were performed under the same conditions of water quality, temperature, photoperiod, and feeding as described above. All aquaria were cleaned, and water was completely replaced once a week. The experimental period comprised 105 days for Group A, 90 days for Group B, and 120 days for Group C, during which animals were weighed (precision 0.01 mg) and their mortality recorded every 2 weeks.

Morphological and histological examination

At the end of each of the three experiments, females were weighed (precision: 0.1 mg), and after being cold-anaesthetized at −20 °C for 15 min, their carapace was removed and the ovaries were inspected to evaluate their relative size, structure, and color. The stage of ovarian development was assessed using a four-stage ovary color pattern as follows: transparent (stage I), yellowish to orange (stage II), orange with some olive green oocytes (stage III), and fully olive green (stage IV), according to Vazquez *et al.* (2008).

The ovaries were dissected rapidly, weighed, and fixed in Bouin's solution for 4 h at room temperature. Finally, the tissues were dehydrated and embedded in paraffin. Sections (5–7 μm thick) were stained with hematoxylin-eosin (López Greco *et al.* 2007). At least three slides from each crayfish were examined under light microscope. The presence of primary and secondary oocytes was determined, and the MOD of each category was measured with an 8× Zeiss microscopic ocular lens, calibrated against a Leitz Wetzlar plate with 10-μm spacing on a representative section of each ovary. Only those oocytes with visible nuclei were measured (Vazquez *et al.* 2008; Sánchez De Bock and López Greco 2010).

In the case of Group C, having fully mature ovaries, the MOD was measured under stereoscopic microscope. For this purpose, 10 secondary oocytes were selected per female if the ovary was mature (stage IV), 10 primary oocytes if the ovary was at stage II, and a combination of 10 primary and 10 secondary oocytes if the ovary was at stage III.

Finally, the moisture content of the ovaries was determined. For this purpose, a piece of each female ovary was weighed (wet weight; precision: 0.1 mg) and then oven-dried at 60 °C to a constant weight (dry weight; precision: 0.1 mg).

Statistical analysis

Survival was calculated as the percentage of crayfish alive per aquarium at the end of each experiment.

The formulae used to calculate the indexes related to reproductive parameters were as follows:

Gonadosomatic Index: GSI = 100 (reproductive system weight/animal weight) (Sagi *et al.* 1996).

Proportion of Ovarian Stage X: POS = 100 (number of ovarian stages X/total number of ovarian stages).

Ovarian Moisture Content: MO = [100 (wet weight of the ovarian piece − dry weight of the ovarian piece)]/wet weight of the ovarian piece.

All the indexes described were calculated per aquarium (replicate), except POS that was calculated per treatment.

A one-way analysis of variance (ANOVA) (Sokal and Rohlf 1995) was applied to compare GSI, MOD, MO, survival, and final weight between treatments for each experiment. The Fisher exact test (Sokal and Rohlf 1995) was used to compare POS between treatments for each experiment. The results per treatment are presented as mean ± SE according to Fotedar (2004). All tests were carried out at the 95% significance level.

Results

Reproductive system

At the end of the experimental period, all females from Group A had stage II ovaries. However, two sub-stages were clearly

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distinguished within this stage: (i) sub-stage IIa ovaries, which were pale-cream in color; and (ii) sub-stage IIb ovaries, which were yellow-orange in color (Fig. 1A,B). The proportion of stage IIb ovaries was significantly higher in control females ($P < 0.05$), while the proportion of stage IIa ovaries tended to be higher in DA-injected females, although this difference was not statistically significant ($P > 0.05$; Fig. 2A). The same result was obtained in Group B, but with no statistical significance ($P > 0.05$); control females of this group additionally had ovaries in a more advanced developmental stage (stages III and IV) (Figs 1C,D and 2B). No significant differences in the proportion of ovarian stages were observed between treatments in Group C ($P > 0.05$), with the majority of females having stage IV ovaries (Fig. 2C).

In agreement with these results, the GSI was significantly lower ($P < 0.05$) for DA-injected females from Groups A and B, but it did not differ between treatments in Group C ($P > 0.05$; Fig. 3). In all Groups, the moisture content of the ovaries (MO) was similar in DA-injected and control females ($P > 0.05$; Table 1).

On the other hand, the ovarian histological analysis revealed that the ovaries of females from Group A had only primary oocytes (Fig. 4A,C,E), while stage III and stage IV ovaries of control females from Group B had also secondary oocytes (Fig. 4B). The MOD of primary oocytes was independent of the treatment for both groups of females ($P > 0.05$; Table 1). In Group C, the 75% of females ($n = 14$) had mainly secondary oocytes in their ovaries, while 15% of the remaining females had only primary oocytes. The MOD of the former was similar in both treatments ($P > 0.05$), while the MOD of the latter was significantly higher in DA-injected females ($P < 0.05$; Table 1).

Finally, oocyte atresia was observed in females from Groups A and B, although the frequency of atretic oocytes tended to be higher in DA-injected females from both groups (Fig. 4C–F).

Survival and growth

In Groups A and B, survival was significantly lower ($P < 0.05$) for DA-injected than for control females, while in Group C survival did not differed significantly between treatments ($P > 0.05$; Table 1). Final weight was higher in control females with respect to DA-injected females for Group A ($P < 0.05$), but it was similar between treatments for Groups B and C ($P > 0.05$; Table 1).

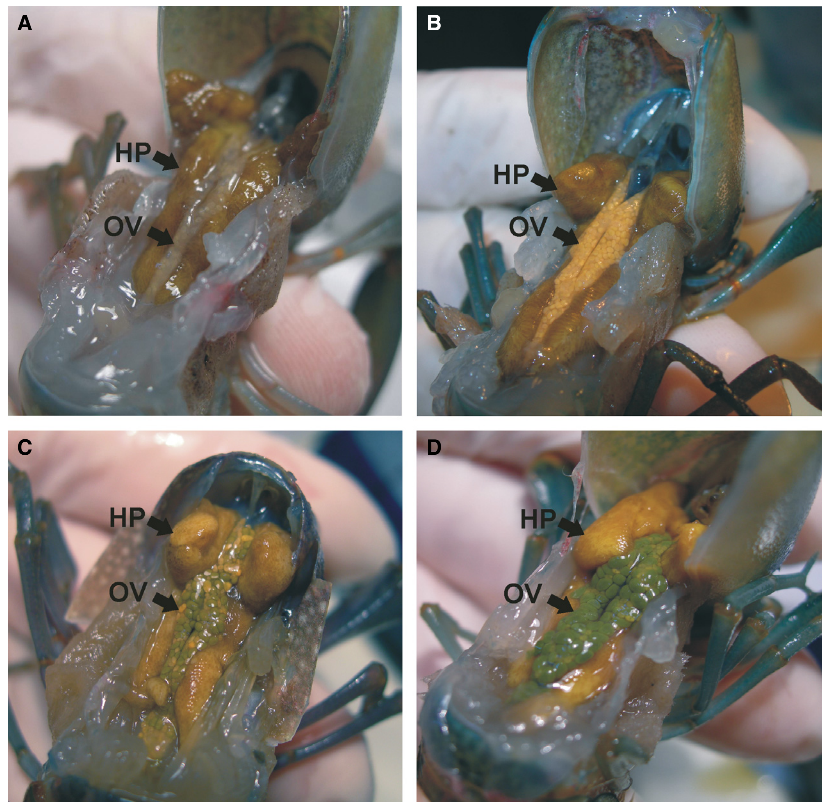

Fig. 1—Ovarian developmental stages of *Cherax quadricarinatus* females. Group A: (**A**) stage II pale-cream ovary of a dopamine (DA)-injected female; (**B**) stage II yellow-orange ovary of a control female. Group B: (**C**) stage III ovary and (**D**) stage IV ovary of control females. HP, hepatopancreas; OV, ovary.

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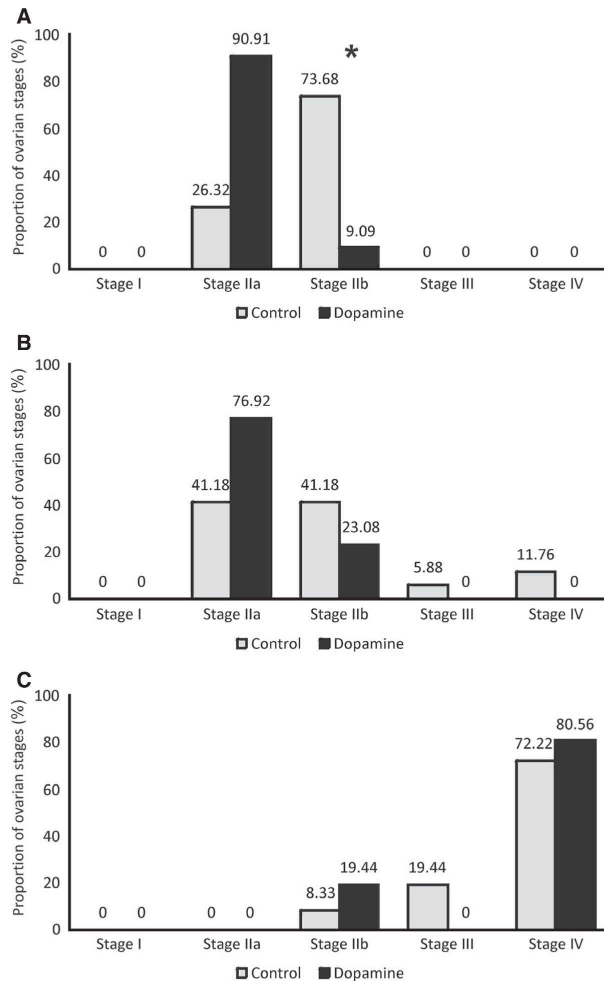

Fig. 2—Proportion of ovarian stages of control (Control treatment) and dopamine (DA)-injected (DA treatment) *Cherax quadricarinatus* females belonging to Group A (**A**), Group B (**B**), or Group C (**C**). The number of females used to calculate this variable per treatment was 19 control and 11 DA-injected females for Group A; 17 control and eight DA-injected females for Group B; and 10 control and 10 DA-injected females for Group C. *Statistically significant differences between treatments within each group of females ($P < 0.05$).

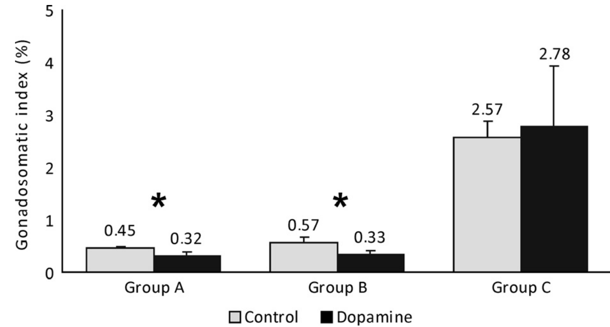

Fig. 3—Gonadosomatic index of control (Control treatment) and dopamine (DA)-injected (DA treatment) *Cherax quadricarinatus* females belonging to Group A, Group B, or Group C. This variable was calculated per treatment as the average of three replicate values ($N = 3$), with each replicate being represented by an aquarium. *Statistically significant differences between treatments within each group of females ($P < 0.05$).

Discussion

In the present study, a lower GSI value and a higher proportion of less developed ovaries was observed in DA-injected females in relation to control females in a weight range of 4–14 g (Groups A and B). In both groups, the MOD of primary oocytes was similar between treatments and similar to the value reported by Vazquez *et al.* (2008) when characterizing the female reproductive system of *C. quadricarinatus*. This result, together with the fact that the moisture content of the ovaries was similar in both treatments, suggests that the lower GSI value observed in DA-injected females from Group A would have been due to a lower number of oocytes per ovary, which would demonstrate in turn an effect of DA administration on the early phases of ovarian growth. In Group B, the higher GSI value of control females was in accordance with the presence in these females of mature ovaries with secondary oocytes, while the absence of stage III and stage IV ovaries in DA-injected females from the same group would demonstrate a certain retardant effect of DA on the onset of ovarian maturation.

On the other hand, DA-injected females from both groups A and B showed a tendency toward a higher proportion of oocyte atresia, a phenomenon previously described for other decapod crustaceans under captivity conditions (Sarojini *et al.* 1995d) or exposed to different pollutants (Kogan *et al.* 2000; Peixoto *et al.* 2005). This is the first report on the atretic effect of a neurotransmitter (DA) administered exogenously (by injection) on the oocytes of a decapod crustacean ovary.

In the case of Group C (females with an initial weight of 14 g), DA injection had no inhibitory effect on ovarian maturation, because the animals treated with this neurotransmitter had the same proportion of mature ovaries, GSI and MOD of secondary oocytes than control females. These results coincide with those obtained by Sarojini *et al.* (1995c) and Sainath and Sreenivasula Reddy (2011) after injecting DA to females of *P. clarkii* and *O. senex senex*, respectively. However, the similar MOD of secondary oocytes found in both treatments of the present study seems to contradict the result obtained by Tinikul *et al.* (2009b), who detected decreased oocyte diameters in DA-injected females of the freshwater prawn *M. rosenbergii* as compared to the control group. Taking into account that the inhibitory effect of DA on vitellogenin synthesis has shown to be dose dependent in *M. rosenbergii* (Chen *et al.* 2003), a possible explanation for the results obtained in the present study is that the concentration of DA used was not high enough to produce a detectable effect. However, the concentration injected per animal was similar to the one used in

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Table 1 Effect of injection of dopamine on survival, growth, and ovarian development in *Cherax quadricarinatus* females after 105 (Group A), 90 (Group B), and 120 (Group C) days

	Group A		Group B		Group C	
Parameter	C	DOPA	C	DOPA	C	DOPA
Initial number	21	21	18	18	12	12
Final number	19	11	17	8	10	10
Survival (%)	90.48 ± 4.76^a	52.38 ± 4.76^b	94.44 ± 5.56^a	44.44 ± 5.56^b	83.33 ± 8.33^a	83.33 ± 8.33^a
Initial weight (g)	4.14 ± 0.02^a	4.16 ± 0.01^a	9.63 ± 0.09^a	9.68 ± 0.12^a	13.98 ± 0.08^a	13.99 ± 0.05^a
Final weight (g)	9.30 ± 0.10^a	7.96 ± 0.52^b	14.47 ± 1.21^a	14.68 ± 0.19^a	21.96 ± 2.91^a	21.71 ± 0.62^a
MO (%)	96.34 ± 0.43^a	97.43 ± 0.33^a	88.84 ± 2.49^a	95.10 ± 0.51^a	61.48 ± 3.21^a	68.87 ± 8.09^a
MOD of primary oocytes (μm)	221.10 ± 2.73^a	210.32 ± 5.79^a	231.43 ± 9.43^a	237.39 ± 37.15^a	283.98 ± 1.27^a	358.67 ± 5.93^b
MOD of secondary oocytes (μm)	–	–	604.70 ± 91.58	–	994.09 ± 16.02^a	893.96 ± 24.68^a

MO, moisture content per ovary; MOD, mean oocyte diameter.
Comparisons were made between the Control (C) and the Dopamine (DOPA) treatments within each Group of females. The value of each of the variables informed was calculated per treatment as the average of three replicate values ($N = 3$), with each replicate being represented by an aquarium. Values (±SE) with the same superscript are not different ($P > 0.05$).

previous studies, in which it provoked clear effects in short experimental periods and in females of a similar and even higher weight than females from Group C (Sarojini *et al.* 1995c; Chen *et al.* 2003; Tinikul *et al.* 2009b). Differences in the experimental periods, in the initial size of the animals, and/or possible intraspecific differences may also explain the discrepancy with the results of all mentioned studies.

An alternative explanation for the absence of DA effect on ovarian maturation of females from Group C is that the hormonal cascade that triggers secondary vitellogenesis could have been already induced at the onset of the experiment, with exogenous DA being unable to inhibit such process. The physiological context that allowed secondary vitellogenesis to occur [high levels of GSH and minimum levels of GIH (Adiyodi and Subramonian, 1983; Tsukimura, 2001)] could have been associated with a low responsiveness of the thoracic ganglion to DA, owing to a possible downregulation of their receptors, or to any change in the intracellular signal transduction mechanism, as proposed by Cahansky *et al.* (2011). Under this possible physiological context of females from Group C, exogenous DA would have had no effect, independently of the dose administered. In this sense, it is highly improbable that secondary vitellogenesis would have been already induced in females from Group B at the onset of the experiment, because the majority of control females presented immature ovaries at the end of it. Hence, the physiological context of these females would have been different to that of Group C, allowing an inhibitory effect of DA on ovarian maturation.

With respect to somatic growth, the inhibitory effect of DA on ovarian growth and maturation of females from Groups A and B did not result in any increase in body weight, as it would have been expected taking into account that ovarian development is proposed to compete with somatic growth from an energetic point of view (Conan 1985; Hartnoll 1985; Provenzano 1985; Sokol 1988; Nelson 1991). In particular, the antagonistic relationship between growth and reproduction is apparent in *C. quadricarinatus* from the alternating trends of spawning and somatic molting events, as demonstrated by Barki *et al.* (1997) under laboratory conditions. Moreover, the reduced body weight observed in the current study in some DA-injected females could be related to the fact that in decapod crustaceans, DA is involved in somatic motor control and pyloric rhythm at the stomatogastric ganglion (Miller *et al.* 1985; Flamm and Harris-Warrick 1986a,b; Martinez *et al.* 1988; Barthe *et al.* 1989; Knotz and Mercier 1995). Probably as a result of this, immediately after injecting DA to juvenile females, certain motor alterations were observed, such as decreased locomotor activity and increased pleopod beating, as previously reported for other decapod species (Martinez *et al.* 1988; Barthe *et al.* 1989). Such alterations were detected in all the animals when injected with DA, persisting during 30 min approximately, after which they completely disappeared. As a consequence of these motor alterations, the animals did not feed in the hours following DA injection. To minimize the possible effects of such abnormal behavior on growth, all crayfish were fed early in the morning, injected at noon, and fed again in the evening. Nevertheless, uneaten food was occasionally detected in the aquaria corresponding to the DA treatment the day following the injection day. Hence, it is possible that food consumption and gastrointestinal function would have been negatively affected by DA injection, with negative consequences in the final weight of females from this treatment.

In summary, the present study reports for the first time the effects of a long-term administration of DA on the ovarian development of juvenile females of a decapod crustacean. DA affected negatively the ovarian growth in a body weight ranging from 4 to 14 g, mainly in terms of both a slower ovarian maturation and a reduced GSI.

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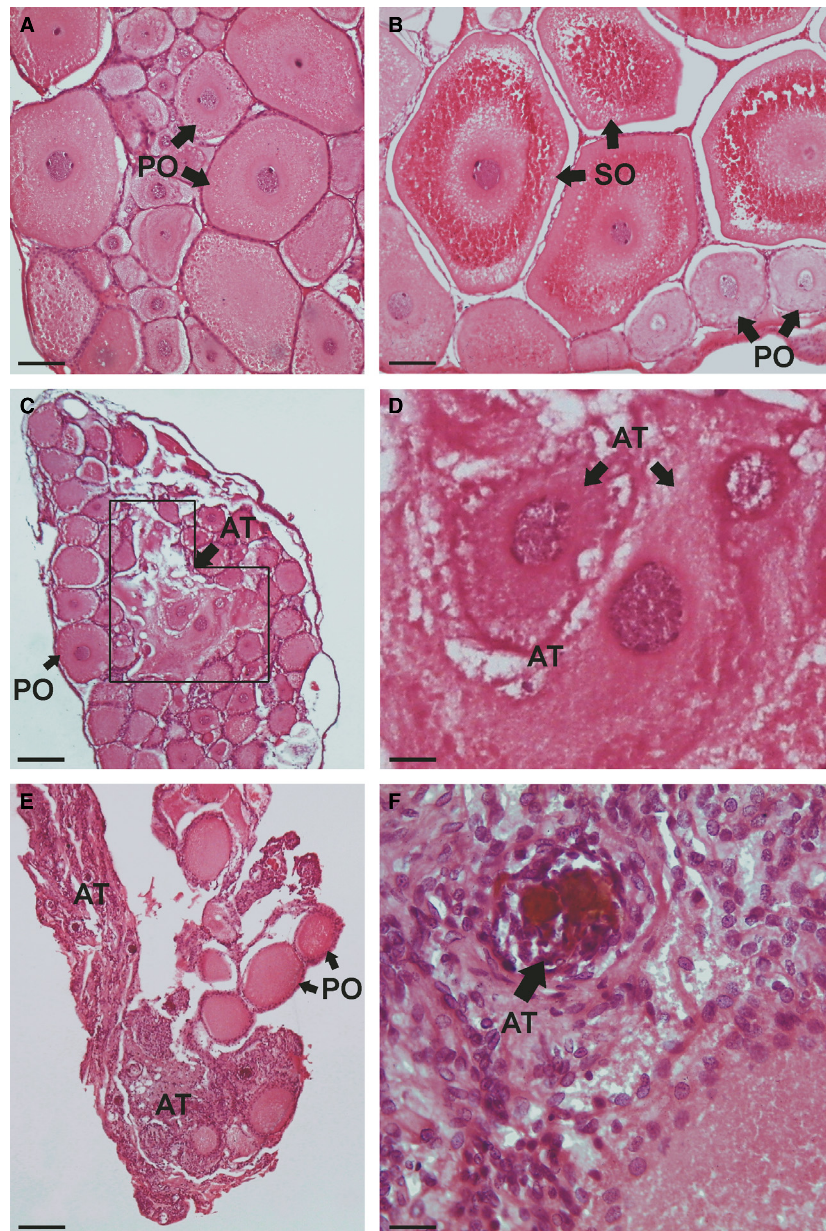

Fig. 4—Histological structure of the ovaries of *Cherax quadricarinatus* females from Groups A and B, stained with hematoxylin-eosin. —**A**. Primary oocytes and (**B**) secondary oocytes of control females from Group B. —**C**. General view and (**D**) detail of the ovary of a dopamine (DA)-injected female from Group A, showing early oocyte atresia. —**E**. General view and (**F**) detail of the ovary of a DA-injected female from Group B, showing advanced oocyte atresia. Scale bars: (**A**), (**B**), (**D**) and (**F**) = 130 μm; (**C**) and (**E**) = 220 μm. AT, oocyte atresia; PO, primary oocyte; SO, secondary oocyte.

Acknowledgements

The work reported in this paper is part of Carolina Tropea's postgraduate scholarship (CONICET) and PhD Thesis (University of Buenos Aires, Argentina). Laura S. López Greco is grateful to Agencia Nacional de Promoción Científica y Tecnológica (PICT 2004, project 953, and PICT 2007, project 01187), CONICET (PIP 2009–2011, number 129) and UBACYT (projects X458 and 20020100100003) that funded this research. We are also grateful to Carlos Anselmi (Farm Las Golondrinas) for providing the crayfish.

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