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In Vivo Effects of Dopamine and Dopaminergic Antagonists on Testicular Maturation in the Red Swamp Crayfish, *Procambarus clarkii*

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Abstract. *In vivo,* dopamine (DA) inhibits testicular maturation in the red swamp crayfish, *Procambarus clarkii.* Crayfish given DA injections had a smaller testicular index, smaller testicular lobes, fewer mature sperm, and less-well-developed androgenic glands than did the control crayfish given physiological saline. Males administered 5-hydroxytryptamine (5-HT) or a DA receptor blocker, spiperone or pimozide, showed enhanced testicular maturation and more highly developed androgenic glands than did the control crayfish. When equimolar amounts of 5-HT and DA were co-injected, the actions of DA and 5-HT were found to be antagonistic. These results can be explained by assuming not only that 5-HT triggers release of the gonad-stimulating hormone (GSH) but that DA (a) triggers release of the gonad-inhibiting hormone (GIH), (b) inhibits GSH release, or (c) does both (a) and (b), with GSH and GIH affecting the androgenic glands directly, thereby regulating release of the androgenic gland hormone that has the well-established role of stimulating testicular maturation and spermatogenesis.

Introduction

Biogenic amines function as neurotransmitters in a wide array of animals (Werman, 1966; Gerschenfeld, 1973; Fingerman, 1985). Among the demonstrated roles of at least some of the biogenic amines in crustaceans is regulation of release of neurohormones (Fingerman and Nagabhushanam, 1992; Fingerman *et al.,* 1994).

The presence of the biogenic amines 5-hydroxytryptamine (5-HT) and dopamine (DA) in the nervous systems of crustaceans, including crayfishes, is well established. 5HT-like immunoreactivity in the central nervous system of the red swamp crayfish *Procambarus clarkii,* the species used in the present study, was demonstrated by several investigators (Fujii and Takeda, 1988; Aréchiga *et al.,* 1990; Real and Czternasty, 1990). In addition, 5-HT has been identified and quantitatively measured by high performance liquid chromatography (HPLC) in *Procambarus clarkii* by Kulkarni and Fingerman (1992). Using the crab *Carcinus maenas,* Kerkut *et al.* (1966) provided the first convincing evidence for the existence of DA in the nervous system of a crustacean. Neurons with DA-like immunoreactivity have been visualized in the crayfish *Orconectes limosus* (Elekes *et al.,* 1988), the lobster *Homarus gammarus* (Barthe *et al.,* 1989), and *Procambarus clarkii* (Mercier *et al.,* 1991). By use of HPLC, Elofsson *et al.* (1982) showed the presence of DA in the nervous system of the crayfish *Pacifastacus leniusculus.*

In decapod crustaceans the major neuroendocrine component of the eyestalk, the medulla terminalis X-organ-sinus gland complex, is the source of the gonad-inhibiting hormone (GIH) (Panouse, 1943). In contrast, a gonad-stimulating hormone (GSH) is present in the brain and thoracic ganglia (Otsu, 1960, 1963; Eastman-Reks and Fingerman, 1984). Data from this laboratory provide the basis for the hypothesis that 5-HT triggers release of GSH in both sexes of the fiddler crab *Uca pugilator* (Richardson *et al.,* 1991; Sarojini *et al.,* 1993) and in *Procambarus clarkii* (Kulkarni and Fingerman, 1992; Sarojini *et al.* 1994). On the other hand, DA has so far been found to antagonize the gonad-stimulating action of 5-HT in females of *Procambarus clarkii* (Sarojini *et al.,* 1995a) and in males of *Uca pugilator* (Sarojini *et al.,* 1995b).

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In male crustaceans, in addition to the two neurohormones, GSH and GIH, the androgenic gland hormone (AGH) has a major role in the control of spermatogenesis. The function of the androgenic gland in controlling development and maturation of the reproductive system and secondary sexual characteristics in male crustaceans was first described by Charniaux-Cotton (1954). Initiation of spermatogenesis is due to circulating AGH (Payen, 1973). Spermatogenesis stops when the androgenic glands are removed (Charniaux-Cotton, 1964; Puckett, 1964; Nagamine *et al.*, 1980). Removal of both eyestalks, thereby removing the source of GIH, results in hypertrophy of the androgenic glands and precocious spermatogenesis (Meusy, 1965; Demeusy, 1967; Payen *et al.*, 1971). Thus, GIH appears to exert its effect on the testes indirectly, by inhibiting the androgenic glands. On the other hand, a GSH is required to activate the androgenic glands for spermatogenesis to occur (Juchault and Legrand, 1965), a process that Payen (1980) referred to as a positive control of the androgenic glands by a neurohormone. Gupta *et al.* (1989) suggested from their studies of the crab *Paratelphusa hydrodromus* that the inactive phase of the testes is due to an increase in the hemolymph titer of GIH with concomitant decreases in the titers of GSH and AGH.

In view of the facts that 5-HT stimulates gonadal maturation in both male and female *Procambarus clarkii* and DA antagonizes this action of 5-HT in females of this species, this investigation was designed to determine (a) whether DA inhibits testicular maturation in *Procambarus clarkii*, (b) whether 5-HT and DA act antagonistically on gonadal maturation and spermatogenesis in the male crayfish, and (c) whether the androgenic glands will be affected when DA or a dopaminergic receptor blocker is injected. This is the first report that shows injection of DA affects the androgenic glands of any crustacean.

Materials and Methods

Experimental animals

Specimens of the red swamp crayfish, *Procambarus clarkii*, were purchased from a local seafood dealer. In the laboratory they were maintained in freshwater tanks where the water was recirculated constantly through sand filtration units. Male intermolt crayfish with a carapace length of 30–35 mm and a body weight of 11–12 gm were used for these experiments. The crayfish were maintained at a room temperature of 24 ± 2°C, with 12 h of light daily, from 8:00 A.M. to 8:00 P.M., and were fed commercial crayfish food daily.

Drugs

5-HT creatinine sulfate, DA hydrochloride, spiperone, and pimozide were purchased from the Sigma Chemical Company (St. Louis, MO). The drugs were dissolved in crayfish physiological saline (Van Harreveld, 1936). To prepare the stock solution of spiperone a few drops of acetic acid were added to facilitate solubilization. When DA was used 1×10^{-6} mol, 1×10^{-7} mol and 1×10^{-8} mol per crayfish were injected. The amounts of 5-HT, spiperone and pimozide injected were 1×10^{-6} mol per crayfish. The volume injected into each crayfish was 100 μl.

The testicular index (TI) was determined for each crayfish used in these experiments according to the standard formula:

$$\text{TI} = \frac{\text{Weight of the testes}}{\text{Weight of the crayfish}} \times 100$$

The testes and androgenic glands were removed from each of the crayfish used in these experiments after the crayfish were weighed at the time of sacrifice. When these organs were removed the testes were weighed. The testes and androgenic glands were then fixed for 24 h in Bouin's fluid, dehydrated in an alcoholic series, and embedded in paraffin (m.p. 56°–58°C). Sections (7 μm) were cut and stained with Delafield's hematoxylin followed by counterstaining with alcoholic eosin (Bancroft and Stevens, 1982). The diameters of 50 testicular follicles (μm) in the testes of each male were measured by use of a compound microscope fitted with an ocular micrometer. The number of mature sperm per follicle was also determined. The diameters (μm) of 50 cells in each androgenic gland were likewise measured. The experiments were performed twice and the averaged results are presented in the figures where each value represents the mean for 20 crayfish, except for bars IC and SC in Figure 7 which, as we'll explain below, represent the means for 40 crayfish. The data were analyzed by means of Student's *t*-test with significance set at the 95% confidence interval. Standard errors of the means were also calculated.

Results

Effect of DA on the testes

To determine the response of the testes to DA, each time the experiment was done 50 male crayfish were divided into five groups of 10 each. The first group served as the initial control, and this group of crayfish, which received no treatment, was sacrificed on the first day of the experiment. The initial control crayfish were weighed, and then their testes and androgenic glands were dissected out. Then, as stated above, the paired testes were weighed, and the testes and androgenic glands were fixed in Bouin's fluid. A simultaneous control group received only physiological saline in 100 μl doses. The last three groups ran concurrently with the simultaneous control group and

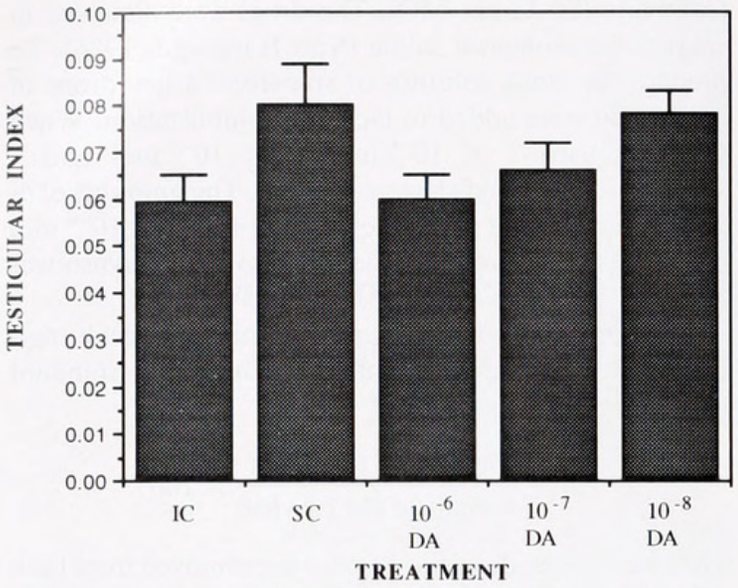

Figure 1. Effect of different doses (1×10^{-8}, 1×10^{-7}, and 1×10^{-6} mol per crayfish) of dopamine (DA) on the mean testicular index of the crayfish, *Procambarus clarkii*. IC, initial control; SC, simultaneous control. Error bars are SEM. Bar SC is significantly ($P < 0.05$) larger than bars IC, 10^{-6} DA, and 10^{-7} DA. Bar 10^{-8} DA is significantly ($P < 0.05$) larger than bar 10^{-6} DA.

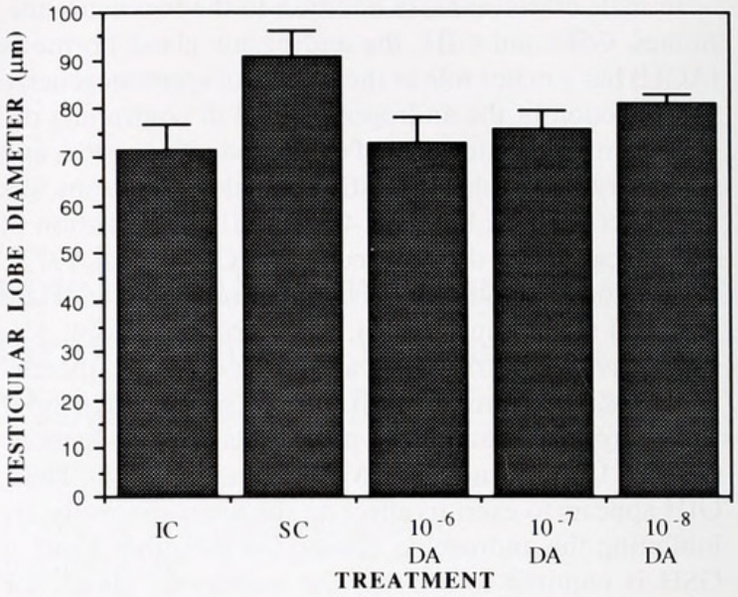

Figure 2. Effect of different doses (1×10^{-8}, 1×10^{-7}, and 1×10^{-6} mol per crayfish) of dopamine (DA) on the mean testicular lobe diameter of the crayfish, *Procambarus clarkii*. IC, initial control; SC, simultaneous control. Error bars are SEM. Bar SC is significantly larger ($P < 0.05$) than bars IC, 10^{-6} DA, and 10^{-7} DA.

received 1×10^{-6} mol, 1×10^{-7} mol, and 1×10^{-8} mol DA per crayfish respectively in 100 μl doses. Injections were administered on the 1st, 5th, and 10th days. The simultaneous control group and those given DA were sacrificed on the 15th day and processed in the same manner as the initial control group.

The TI and mean testicular lobe diameter of the simultaneous control group were significantly larger than the corresponding values of the initial control group, showing that during the 15 days of the experiments the testes were undergoing maturation (Figs. 1, 2). Furthermore, the simultaneous control testes contained mature sperm whereas the initial control testes had none (Fig. 3). The TI and mean testicular lobe diameter of the crayfish that received 1×10^{-6} mol DA injections were significantly smaller than the corresponding values for the simultaneous control crayfish that received only physiological saline. Furthermore, there were no mature sperm in the testicular lobes of the crayfish that received the injections of 1×10^{-6} mol DA in contrast to the simultaneous control crayfish. The crayfish that received injections of the two lower doses of DA (1×10^{-7} mol and 1×10^{-8} mol) also had a smaller TI and mean testicular lobe diameter than the simultaneous control crayfish, but only the difference between the testicular lobe diameter of the simultaneous controls and the crayfish that received injections of 1×10^{-7} mol DA was statistically significant. The testes of the crayfish that received injections of the two lower doses of DA contained mature sperm, but significantly fewer than in the simultaneous control group.

It is evident from Figures 1–3 that DA inhibited testicular maturation. The responses to the three concentrations of DA used strongly suggest that this inhibition is dose-related, as in Figures 1 and 3 where the inhibition produced by 1×10^{-6} mol DA per crayfish is significantly greater than that produced by 1×10^{-8} mol DA per crayfish.

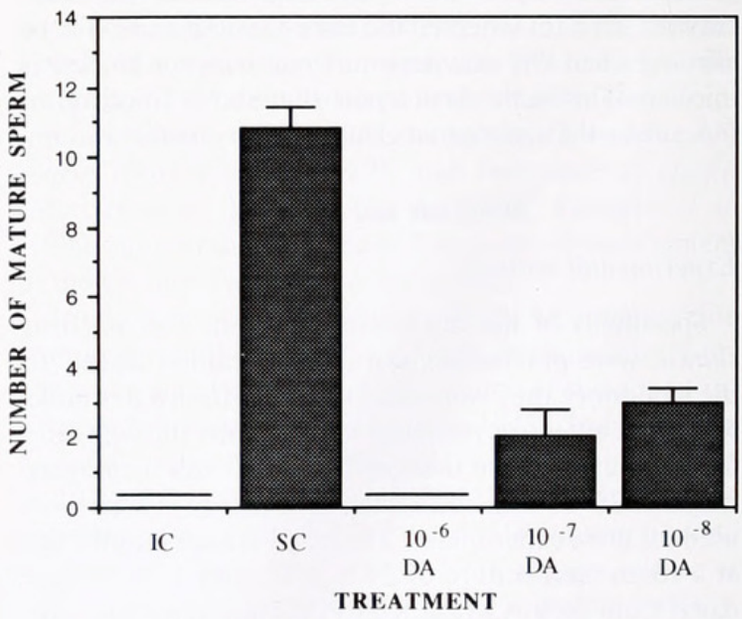

Figure 3. Effect of different doses (1×10^{-8}, 1×10^{-7}, and 1×10^{-6} mol per crayfish) of dopamine (DA) on the mean number of mature sperm per follicle in the testes of the crayfish, *Procambarus clarkii*. IC, initial control; SC, simultaneous control. Error bars are SEM. Bar SC is significantly larger ($P < 0.05$) than bars IC, 10^{-6} DA, 10^{-7} DA, and 10^{-8} DA. Bars 10^{-7} DA and 10^{-8} DA are significantly ($P < 0.05$) larger than bar 10^{-6} DA.

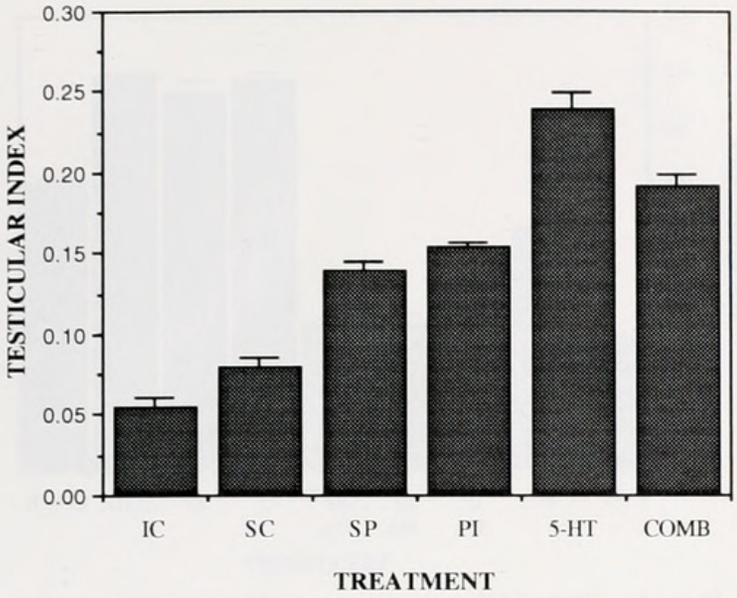

Figure 4. Effect of different treatments on the mean testicular index of the crayfish, *Procambarus clarkii*. IC, initial control; SC, simultaneous control; SP, 1×10^{-6} mol spiperone per crayfish; PI, 1×10^{-6} mol pimozide per crayfish; 5-HT, 1×10^{-6} mol 5-HT per crayfish; COMB, combination of 1×10^{-6} mol DA per crayfish + 1×10^{-6} mol 5-HT per crayfish. Error bars are SEM. Bar SC is significantly ($P < 0.05$) larger than bar IC, but bar SC is significantly ($P < 0.05$) smaller than bars SP, PI, 5-HT, and COMB. Bar 5-HT is significantly ($P < 0.05$) larger than bar COMB.

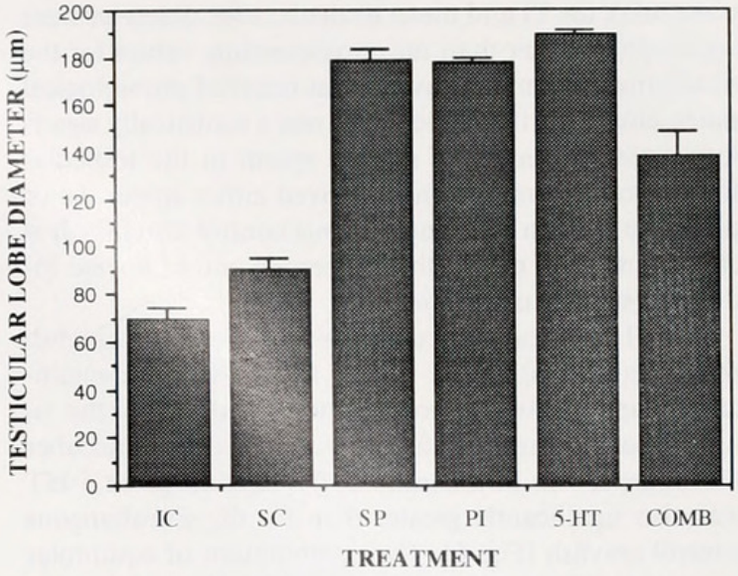

Figure 5. Effect of different treatments on the mean testicular lobe diameter of the crayfish, *Procambarus clarkii*. IC, initial control; SC, simultaneous control; SP, 1×10^{-6} mol spiperone per crayfish; PI, 1×10^{-6} mol pimozide; 5-HT, 1×10^{-6} mol 5-HT per crayfish; COMB, combination of 1×10^{-6} mol DA per crayfish + 1×10^{-6} mol 5-HT per crayfish. Error bars are SEM. Bar SC is significantly ($P < 0.05$) larger than bar IC, but bar SC is significantly ($P < 0.05$) smaller than bars SP, PI, 5-HT, and COMB. Bar 5-HT is significantly ($P < 0.05$) larger than bar COMB.

Effects of the DA receptor blockers spiperone and pimozide, 5-HT alone, and 5-HT in combination with DA on the testes

For each replicate of this set of experiments, 6 groups of 10 crayfish were selected from the stock. One group served as the initial control; the crayfish of this group were treated in the same way as the initial control crayfish of the DA dose-response experiment. The crayfish in the simultaneous control group received physiological saline in 100 μl doses. Two groups received 1×10^{-6} mol of the DA receptor blockers spiperone and pimozide respectively in 100 μl doses. Another group received 1×10^{-6} mol of 5-HT per crayfish in 100-μl doses and the last group received 1×10^{-6} mol DA in 50-μl doses + 1×10^{-6} mol 5-HT in 50-μl doses per crayfish, respectively. Injections were administered on the 1st, 5th, and 10th days. All the crayfish that received injections were sacrificed on the 15th day, and their testes were processed in the same manner as those of the initial control group.

As in the previous experiment, the TI and testicular lobe diameter of the simultaneous control crayfish were significantly larger than the corresponding values for the initial control group (Figs. 4, 5) and, although the initial control testes had no mature sperm, the simultaneous control testes did have some mature sperm (Fig. 6). For the crayfish that each received 100-μl injections of 1×10^{-6} mol of either of the DA receptor blockers (spiperone or

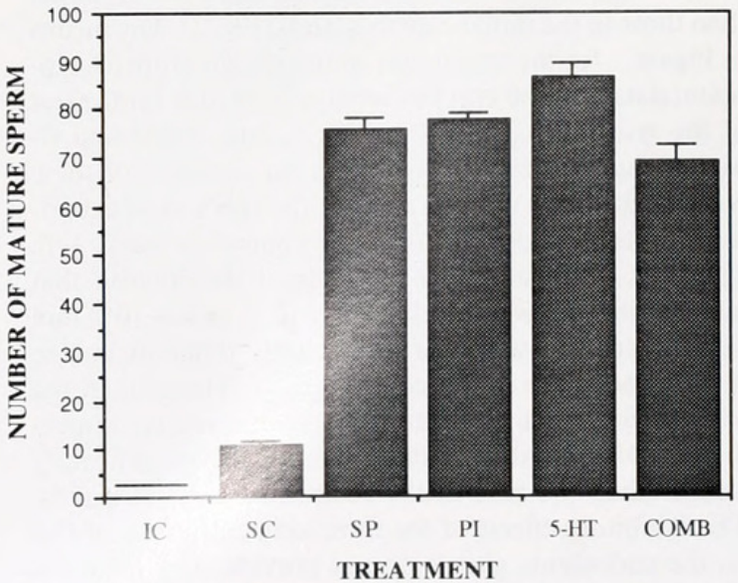

Figure 6. Effect of different treatments on the mean number of mature sperm per follicle in the testes of the crayfish, *Procambarus clarkii*. IC, initial control; SC, simultaneous control; SP, 1×10^{-6} mol spiperone per crayfish; PI, 1×10^{-6} mol pimozide per crayfish; 5-HT, 1×10^{-6} mol 5-HT per crayfish; COMB, combination of 1×10^{-6} mol DA per crayfish + 1×10^{-6} mol 5-HT per crayfish. Error bars are SEM. Bar SC is significantly ($P < 0.05$) larger than bar IC, but bar SC is significantly ($P < 0.05$) smaller than bars SP, PI, 5-HT, and COMB. Bar 5-HT is significantly ($P < 0.05$) larger than bar COMB.

pimozide), the TI and mean testicular lobe diameter were significantly larger than the corresponding values for the simultaneous control crayfish that received physiological saline alone. Furthermore, there was a statistically significant greater number of mature sperm in the testicular follicles of the crayfish that received either spiperone or pimozide than in the simultaneous control crayfish. It is clear from these results that spiperone and pimozide induced testicular maturation.

The TI and mean testicular lobe diameter of the crayfish that received 100 μl of 1×10^{-6} mol 5-HT were significantly larger than the corresponding values for the simultaneous control crayfish (Figs. 4, 5), and the number of mature sperm in the testes of the crayfish given 5-HT was also significantly greater than for the simultaneous control crayfish (Fig. 6). The combination of equimolar amounts of DA and 5-HT produced significant increases in the TI, testicular lobe diameter, and sperm count but significantly less than did 5-HT alone. These results show that DA and 5-HT act antagonistically, but DA was not able to inhibit completely the stimulatory action of 5-HT.

Effects of DA, DA antagonists, 5-HT alone, and 5-HT in combination with DA on the androgenic gland

The androgenic glands of the initial control crayfish consisted of only a few cords of cells closely associated with the vas deferens. These cells had a thin rim of homogeneous cytoplasm around the nucleus. The cells of the simultaneous control crayfish were significantly larger than those in the initial control glands (Fig. 7). The means in Figure 7 for the initial and simultaneous controls represent data from 40 crayfish versus 20 crayfish for the rest of the groups because the means for the initial and simultaneous controls are based on the averages of these controls from the crayfish used in the two sets of experiments that provided the data for Figures 1–3 and 4–6. The cells of the androgenic glands of the crayfish that received injections of 1×10^{-6}, 1×10^{-7}, or 1×10^{-8} mol DA per crayfish were not significantly different in size from those of the initial control group. The cells in the androgenic glands of all the crayfish that received injections of DA, regardless of the dose used, were significantly smaller than the cells in the concurrent control glands. The inhibitory effects of the three concentrations of DA on the androgenic glands do not provide clear evidence of a dose-related response, although the highest concentration produced somewhat more inhibition than did the two lesser doses. The androgenic glands of crayfish that received a DA receptor blocker, spiperone or pimozide, 5-HT alone or 5-HT in combination with DA showed significantly greater development of their androgenic glands over the initial and simultaneous controls. The cytoplasm in these enlarged glands was more dense and granular than in either control group. As with the testes, while the combination of 5-HT and DA produced significant growth of the androgenic glands, this growth was significantly less than that produced by 5-HT alone, additional evidence of antagonistic actions of DA and 5-HT on the reproductive system of male red swamp crayfish.

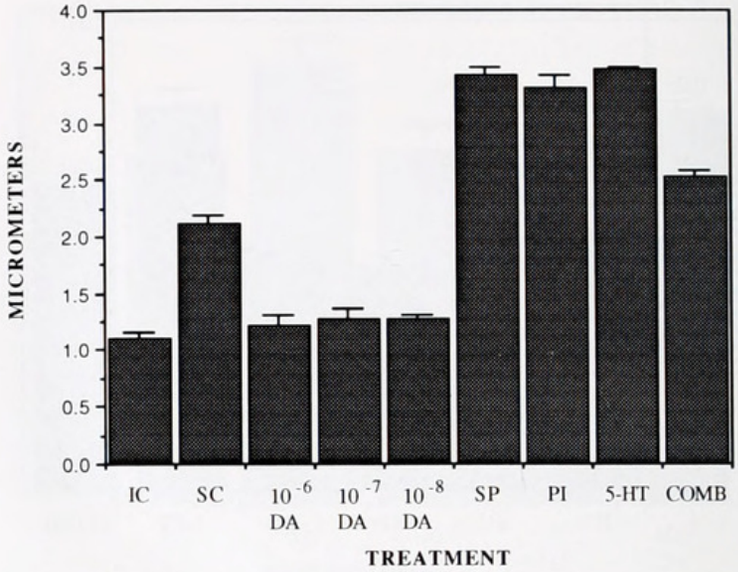

Figure 7. Effect of different treatments on the mean cell size in the androgenic glands of the crayfish, *Procambarus clarkii*. IC, initial control; SC, simultaneous control; 10^{-6} DA, 1×10^{-6} mol DA per crayfish; 10^{-7} DA, 1×10^{-7} mol DA per crayfish; 10^{-8} DA, 1×10^{-8} mol DA per crayfish; SP, 1×10^{-6} mol spiperone per crayfish; PI, 1×10^{-6} mol pimozide per crayfish; 5-HT, 1×10^{-6} mol 5-HT per crayfish; COMB, combination of 1×10^{-6} mol DA per crayfish + 1×10^{-6} mol 5-HT per crayfish. Error bars are SEM. Bar SC is significantly ($P < 0.05$) larger than bars IC, 10^{-6} DA, 10^{-7} DA, and 10^{-8} DA, but bar SC is significantly ($P < 0.05$) smaller than bars SP, PI, 5-HT, and COMB. Bar 5-HT is significantly ($P < 0.05$) larger than bar COMB.

Discussion

The present study demonstrates for the first time in a crayfish an inhibitory action of DA on the testes. Furthermore, this is the first report of the effect of DA and any of its antagonists on the androgenic glands of any crustacean. DA alone inhibited testicular and androgenic gland maturation (Figs. 1–3, 7). On the other hand, 5-HT and the DA receptor blockers spiperone and pimozide induced testicular and androgenic gland maturation (Figs. 4–7).

In *Procambarus clarkii*, as stated above, gonadal maturation is regulated by both stimulatory and inhibitory neurohormones, maturation being stimulated by GSH from the brain and thoracic ganglia and inhibited by GIH from the eyestalk neuroendocrine system. Our previous studies with *Procambarus clarkii* (Sarojini *et al.* 1993, 1994) showed that 5-HT stimulates gonadal maturation in males and females, presumably by stimulating GSH

release and that DA inhibits ovarian development. The evidence for 5-HT and DA presence in the nervous systems of crayfish (Fujii and Takeda, 1988; Aréchiga *et al.* 1990; Real and Czternasty, 1990; Mercier *et al.*, 1991; Kulkarni and Fingerman, 1992) was already demonstrated.

The roles of DA and 5-HT in regulation of gonadal maturation in vertebrates is documented. Goldfish, *Carassius auratus,* fed the DA agonist apomorphine had elevated plasma levels of growth hormone whereas the circulating levels of gonadotropic hormone were reduced (Wong *et al.*, 1993). Long-term feeding of goldfish with apomorphine induced significant increases in both the body weight and length. 5-HT stimulates gonadotropic hormone release in the goldfish (Somoza *et al.*, 1988; Somoza and Peter, 1991). This effect of 5-HT may be due to direct action on the gonadotrophs or to inhibition of DA release from nerve terminals in the pars distalis. DA inhibits release of this gonadotropic hormone (Yu and Peter, 1992). Similarly, DA appears to inhibit luteinizing hormone release in the frog, *Rana temporaria* (Sotowska-Brochocka *et al.*, 1994).

The crayfish that received 5-HT alone had a larger TI and mean testicular lobe diameter and also had more mature sperm in their testicular lobes than did the simultaneous control group (Figs. 4–6) which is consistent with the earlier results of Sarojini *et al.* (1994). The crayfish that received 5-HT in combination with DA had a significantly larger TI and mean testicular lobe diameter, and also a greater number of mature sperm, when compared with the simultaneous controls (Figs. 4–6), but all three values were significantly smaller than the corresponding values of the crayfish given 5-HT alone. The DA in the mixture was not able to antagonize fully the stimulatory action of the 5-HT. This antagonism between the effects produced by 5-HT and DA on the testes is reminiscent of that seen with the erythrophores of *Uca pugilator* where the pigment-dispersing effect of 5-HT and the pigment-concentrating effect of DA were reduced when mixtures of 5-HT and DA were co-injected (Fingerman and Fingerman, 1977).

The data obtained with the DA antagonists used in the present study support the conclusion that DA inhibits testicular maturation. Both spiperone and pimozide produced testicular maturation (Figs. 4–6). Presumably, these blockers prevent the action of endogenous DA, hence leading to precocious testicular maturation.

The inhibitory action of DA on the androgenic glands and testes in *Procambarus clarkii* can be explained as follows: DA has an indirect action on the testes and androgenic glands. We hypothesize that DA either (a) stimulates release of GIH from the eyestalk neuroendocrine system, (b) inhibits release of GSH, or (c) does both (a) and (b). Any of these hypothesized actions of DA would result in reduced AGH in the blood, resulting in at least some inhibition of testicular maturation and spermatogenesis. Experiments are currently in progress to evaluate these suggested modes of action of DA. That DA can have a stimulatory role in the release of a neurohormone was shown for the red pigment-concentrating hormone, as reported by Fingerman and Fingerman (1977) and Quackenbush and Fingerman (1984) who performed *in vivo* and *in vitro* experiments on release of this neurohormone with the fiddler crab, *Uca pugilator.* The concentrations of biogenic amines used in these experiments are quite like those injected by other investigators while studying the same species, *Procambarus clarkii.* Livingstone *et al.* (1980) injected 5.7×10^{-6} mol 5-HT and 6.5×10^{-6} mol octopamine per crayfish, and Aréchiga *et al.* (1990) injected 1×10^{-9} to 1×10^{-3} mol 5-HT per crayfish.

Because DA inhibited testicular maturation in *Procambarus clarkii,* it is worth mentioning the potential application of DA analogues in crayfish farming. Supplementing the crayfish diet with long-lasting DA agonists may slow reproductive activity of crayfish and simultaneously lead to enhanced somatic growth.

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