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Physiological effects of methyl farnesoate and pyriproxyfen on wintering female crayfish *Cherax quadricarinatus*

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Abstract

Methyl farnesoate (MF), the predominant juvenile hormone-like compound of crustaceans, was found in the hemolymph of female *Cherax quadricarinatus* crayfish. Administration of MF to *C. quadricarinatus* females during their winter reproductive arrest period had no effect on reproduction; however, it did have a tendency to accelerate molting. However, since MF caused increased mortality (~47% survival in the high MF treatment), we were not able to draw definitive conclusions regarding its physiological affect. In contrast, administration of pyriproxyfen, a juvenile hormone analog, did not cause significant mortality (95% survival in the high pyriproxyfen treatment), although it accumulated in high quantities in the hepatopancreas and, to a lesser extent, muscle tissue, ovaries and gills. The highest dose of pyriproxyfen used in this study, 20 μg/gram animal body weight/week, caused a delay in spawning, which became statistically significant from the seventeenth week. This dose of pyriproxyfen caused a tendency of acceleration of molting without any effect on molt increment. The results of this study show that

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pyriproxyfen does not seem to be toxic to the crayfish, even in relatively high doses, and might affect the energetic balance between molt and reproduction.

Keywords: Reproduction; Methyl farnesoate; Spawning; Molt; Juvenile hormone; Pyriproxyfen; Crustacea; Crayfish; *Cherax quadricarinatus*

1. Introduction

Studies of a large broodstock population of *Cherax quadricarinatus* (von Martens) (Decapoda, Parastacidae), maintained under temperate zone conditions in Israel, showed a seasonal pattern of spring–summer breeding with a decrease (almost to cessation) in spawning during the winter (Barki et al., 1997). It has, therefore, been suggested that in temperate zones induction of spawning during the winter reproductive arrest is important for obtaining juveniles for stocking as soon as water temperature is high enough for growth (Sagi et al., 1997). In commercial hatcheries of stringently controlled crustacean species, notably penaeids, vitellogenesis and spawning are stimulated by removal of the neuroendocrine complex in the eyestalk, comprising the X-organ and the sinus gland (Quackenbush, 1991). Unilateral eyestalk ablation in *C. quadricarinatus* increased spawning activity during the winter reproduction-arrest period in first-time spawners, but did not affect females that had spawned previously (Sagi et al., 1997). However, eyestalk removal is a permanent alteration that ultimately interferes with the organism's capacity to regulate a number of physiological processes and, therefore, is not favorable for use as a standard aquaculture practice.

Since eyestalk removal significantly increased the levels of methyl farnesoate (MF) in the hemolymph of the spider crab *Libinia emarginata* (Laufer et al., 1997), an alternative to eyestalk ablation as a means of induction of winter spawning in *C. quadricarinatus* may be the administration of MF or an MF analog (Laufer, 1992). Direct in vitro experiments have shown that the production of MF in the mandibular organ is negatively regulated by an eyestalk neurohormone, mandibular organ inhibiting hormone (MOIH) (Liu and Laufer, 1996; Wainright et al., 1996). The functions of MF in crustaceans appear to be manifold, with the compound playing a role in the promotion of the molt cycle (Homola and Chang, 1997b; Tamone et al., 1997), in growth regulation (Laufer et al., 1997; Abdu et al., 1998), and in male and female reproduction (Laufer et al., 1992; Laufer et al., 1993; Homola and Chang, 1997b). The role of MF in crustacean reproduction has been reviewed by Laufer et al. (1992), and recent work has shown that exogenous MF stimulates and enhances ovarian maturation in the crayfish *Procambarus clarkii* (Laufer et al., 1998).

Crustaceans have many morphological, developmental, and physiological features in common with insects. In insects, juvenile hormones regulate metamorphosis and reproduction (Riddiford, 1985), and a number of juvenile hormone analogs are known to stimulate reproduction. Such analogs have longer half-life than MF, which is susceptible to the action of specific esterases (Homola and Chang, 1997a; Takac et al., 1997). One of the most potent juvenile hormone analogs is pyriproxyfen, a compound that is known

to affect insect reproduction (Edwards et al., 1993; Cusson et al., 1994) and growth (Bull and Meola, 1993) in a manner similar to that of a juvenile hormone.

In this study, we confirmed the presence of MF in the hemolymph of *C. quadricarinatus* and undertook preliminary testing of its effect on reproductive females during their winter-reproductive arrest period. We also studied the effects of pyriproxyfen on reproduction and growth of wintering female *C. quadricarinatus* crayfish.

2. Materials and methods

2.1. Detection of MF in the hemolymph

Hemolymph samples (2 ml) were withdrawn from the sinuses at the base of the fifth walking legs of *C. quadricarinatus* females. The samples were transferred to Kimax culture tubes, containing 5 ml of acetonitrile and 2 ml of 4% NaCl. A known amount of the nonbiological isomer of MF (*cis–trans* MF) was added to each tube as an internal standard. The samples were extracted twice with 0.5 ml of hexane. The hexane phase was transferred to amber glass vials (1.5 ml), evaporated under nitrogen, resuspended in 50 μl of hexane, and then stored at −20°C. Hemolymph extracts were analyzed for MF by a method using normal-phase HPLC (Laufer et al., 1987) as follows. The entire amount of each extract was injected into a Beckman 126 Solvent module/168 DAD Detector set at 214 and 220 nm. The isomers of MF were separated using a Microsorb-MV silica column (Rainin) (250 × 4.6 mm ID, 5 μm particle size) at 1200–1500 psi, with 1% diethylether/hexane as the running solvent (1 ml/min). The MF peaks were identified by comparison of their retention times with those of external standards, which were run at the beginning and the end of each series of analyses. Peak areas were calculated using Gold Nouveau chromatography software (Beckman, Version 1.7; 1996). The amount of the biological isomer, all-*trans* MF, in each extract was determined by comparing its peak area to that of the internal standard.

2.2. Effect of MF on wintering female crayfish

2.2.1. Animals

"First-time spawners" (Sagi et al., 1997), approximately five months old were used in this experiment. This female population did not contain berried females. The animals weighed 18.84 ± 2.0 g (mean ± S.E.) and had a carapace length of 31.6 ± 1.27 mm. To study the status of the ovaries at the beginning of the reproductive-arrest period, seven females were dissected at the first week of November. For these animals, the average gonado-somatic index (GSI) was 0.06 ± 0.02 and mean oocyte diameter was 415 ± 97 μm.

2.2.2. MF experimental design

Forty five females were divided into three groups (control and two different doses of MF), and each group was subdivided to three different holding tanks, each containing five females and one sexually mature male. The experiment lasted 15 weeks from early

November until late February. Each 120-l holding tank was equipped with a gravel filter and shelters and was maintained under a 14 light:10 dark light regime and a temperature of 27 ± 2.0°C. Each female was individually marked with colored plastic bands glued to its carapace. The tanks were checked daily for molts, and two days after molting the marking on the carapace was renewed. Females were checked for the presence of eggs once a week. The two MF groups were fed wheat grains or commercial feed pellets that had been immersed in MF dissolved in ethanol (as a solvent vehicle) to give approximate doses of 4.5 μg or 9 μg/animal/week. This dosage was obtained by diluting MF in ethanol and then soaking the wheat grains or pellets in the MF solution. The vehicle was then evaporated off in a hood overnight with gentle agitation. The animals were fed three times a week with immersed wheat grains or pellets. The control group was fed wheat grains or commercial feed pellets immersed in vehicle alone (ethanol). At the end of the experiment, the concentration of MF in the hemolymph of females was determined in treatment and control groups.

2.3. Gas chromatograph-mass spectrometry (GC / MS) analysis of pyriproxyfen

Preliminary analysis and identification of pyriproxyfen was performed by GC using a Hewlett-Packard 5890 unit (Hewlett-Packard, Atlanta, GA, USA), and quantitative analysis and confirmation of the analysis was performed by GC 5890 equipped with a DB-5 capillary column (25 m × 0.25 mm I.D. × 0.25 μm film thickness) coupled with a Hewlett-Packard 5989A MS engine and 5971 MSD in EI mode. Pyriproxyfen was donated by Agan Chemical Manufacturers, Israel. Pyriproxyfen identification was accomplished in the SIM mode using ion chromatogram for mass to charge (m/z) ratios 78, 96, 136, 137, 186, 226 and 322. The GC operating conditions were as follows: the source temperature was maintained at 200°C and a quadropole at 100°C; injector temperature was 250°C, and the detector/interface temperature was 280°C. The oven temperature was increased from 80°C to 190°C at a rate of 30°C/min and from 190°C to 280°C at 10°C/min, held 1 min and then increased to 300°C at a rate of 20°C/min and held for 7 min. Quantification of pyriproxyfen was done as follows: fortified samples were prepared by injection of pyriproxyfen into control tissues to create the calibration curves. Fortification levels resulted in a final concentration ranging from 25 to 1000 ppb.

2.4. Extraction of pyriproxyfen from crayfish tissues and pyriproxyfen analysis

Female crayfish to which pyriproxyfen had been administered via their food and untreated females were anesthetized on ice. Two milliliters of hemolymph samples that were withdrawn from the sinuses at the base of the fifth walking legs were added to 4 ml of doubly distilled water and 1 ml of saturated NaCl solution. Samples of muscles, gills, ovaries, and hepatopancreas were dissected out and frozen at −20°C. For each type of tissue, 0.5–4 g of tissue was homogenized with 4 ml of doubly distilled water and 1 ml of saturated NaCl solution. For each type of tissue, pyriproxyfen was extracted twice with 5 ml of *n*-hexane:acetone (9:1 v/v). The combined extracts were concentrated by evaporation, redissolved in 25% methanol in water, and applied to a solid-phase

extraction column. Pyriproxyfen was eluted from the column with hexane, which was evaporated to dryness. The residue was dissolved in 0.1 ml of hexane immediately prior to GC-MS analysis.

2.5. Effect of pyriproxyfen on female crayfish

2.5.1. Animals

The females used for the pyriproxyfen study were "first-time spawners" (see 2.2.1.). The average weight was 35.2 ± 3.3 g, carapace length was 35.6 ± 1.2 mm, GSI was 0.9 ± 0.1 ($n = 15$) and mean oocyte diameter was 1100 ± 250 μm.

2.5.2. Pyriproxyfen experimental design

The females were held in five separate recirculating water systems, one for each treatment. The experiment lasted 17 weeks from November until early March. Each system consisted of four 120-l tanks, equipped with shelters and maintained under ambient light and a temperature of 26 ± 0.5°C to 28 ± 0.7°C. Each tank included five females and one mature male. Each female was individually marked as described for the MF experiment and checked daily for molting and weekly for the presence of eggs. Since eyestalk ablation was found to increase spawning activity of "first-time spawners" (Sagi et al., 1997), one group of females was subjected to unilateral eyestalk ablation (designated "destalked") and served as a positive control. Three other groups were fed wheat grains immersed in pyriproxyfen dissolved in ethanol (as the solvent vehicle) as described below to give approximate doses of 0.2, 2, or 20 μg/gram animal body weight/week. Pyriproxyfen was first dissolved in 500 μl of hexane, and then ethanol was added to the solution to the final desired concentration of pyriproxyfen. The wheat grains were soaked in this solution, which was then evaporated off in a hood overnight with gentle agitation. A negative control group, and the destalked females (positive control) were fed wheat grains immersed in vehicle alone (ethanol). The animals were fed three times a week, twice in a row with the immersed wheat grains (vehicle alone or with pyriproxyfen) and the third time with either food pellets (37% protein, enriched with calcium) or ground carrots or potatoes.

2.6. Statistical analysis

The χ^2 test was used to study the significance of the differences between the different experimental groups.

3. Results

HPLC gave the base-line levels of the biological all-*trans* isomer of MF in the hemolymph of *C. quadricarinatus* females. A representative chromatogram of one such hexane extract is shown in Fig. 1. Since the level of the hormone in the treated groups was approximated double that of the control group (11 ± 2.2 ng/ml vs. 5.1 ± 2.5 ng/ml), it is evident that the MF was indeed taken up by the animals from the feed.

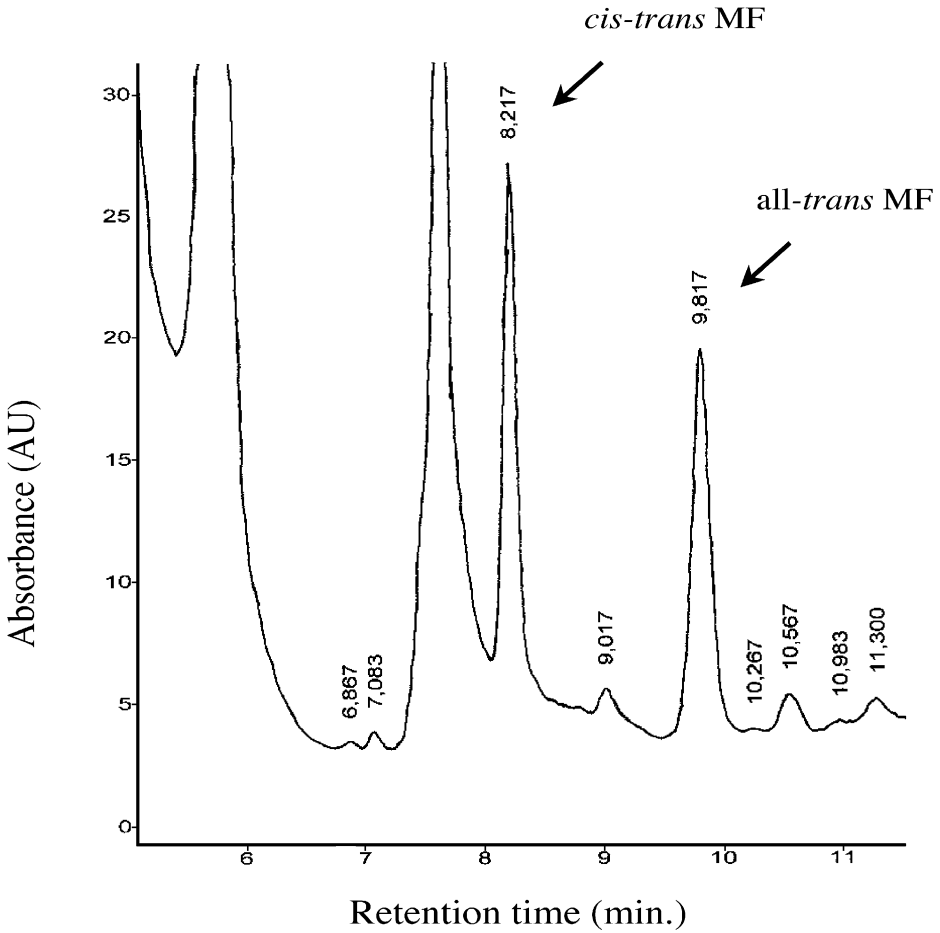

Fig. 1. A representative HPLC chromatogram showing *trans-trans* MF, the biological isomer, in the hemolymph of a *C. quadricarinatus* female. *cis–trans* MF, the nonbiological isomer, served as the internal standard.

Administration of MF to wintering *C. quadricarinatus* females showed a tendency to accelerate molting (Table 1); however, it also resulted in increased animal mortality up

Table 1
Summary of survival and molting events in wintering *C. quadricarinatus* females after 15 weeks of exposure to MF

Treatment (μg MF/animal/week)	Survival (%)	Molting (%)
Control (0)	80.0	58.3
MF I (4.5)	60.0	72.7
MF II (9)	46.7	100

to 53.3% in the high MF treatment (Table 1). The mortality became higher in that group from the ninth week, however, was not significantly different from the two other groups ($p = 0.092$, Fig. 2). No spawning was recorded during the experimental period neither in the two MF-administered groups nor in the control group.

Uptake of pyriproxyfen by the *C. quadricarinatus* females was confirmed by GC/MS. A GC of pyriproxyfen as the external standard showed that this compound is eluted after 13.95 min (Fig. 3A), and the MS of pyriproxyfen showed a typical profile exhibiting selected ion monitoring at m/z 78, 96 and 136 (Fig. 3B). A representative GC of a hexane extract of hepatopancreas from females fed with 20 μg pyriproxyfen/g animal body weight/week showed a peak at 13.94 min (Fig. 3C); this peak exhibited the typical MS profile of pyriproxyfen (Fig. 3D). Table 2 shows that pyriproxyfen was present in significant amounts in the treated females after 119 days of exposure to the compound. The amounts of pyriproxyfen accumulating in the hepatopancreas were higher than those in the muscle tissue, ovaries and gills. Accumulation in the hepatopancreas was dose dependent, being 95.50 ± 4.95, 143.67 ± 34.53 and 561 ± 114 μg of pyriproxyfen/g tissue for the low, medium and high doses, respectively. Pyriproxyfen was also detected in the water of the holding tanks.

The survival rate of the crayfish was high in all treatments (80–95%). It was not affected by the different pyriproxyfen treatments or by eyestalk ablation (Table 3).

Eyestalk ablation of wintering females served as a positive control for the pyriproxyfen experiment. As expected, the destalked females started to spawn as early as the fifth week, and from the eighth week onwards their cumulative % of spawning became significantly higher (χ^2; $p < 0.05$) than in all the other groups (Fig. 4). In the same group, the cumulative % of molts was significantly higher (χ^2; $p < 0.05$) between the sixth week and the ninth week of the experiment (Fig. 5).

From the 12th week onwards, the cumulative % of spawning in the highest-dose pyriproxyfen-treated females was lower than that in the control females, with the difference becoming significant (χ^2; $p < 0.05$) from the 17th week onwards (Fig. 4, Table 3). The females that were treated with the intermediate and lowest pyriproxyfen

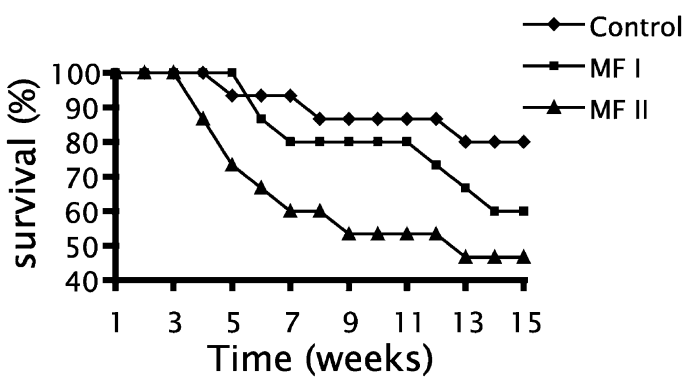

Fig. 2. Effect of MF on survival of *C. quadricarinatus* females during an exposure period of 15 weeks. MF I-4.5 μg/animal/week; MF II-9 μg/animal/week.

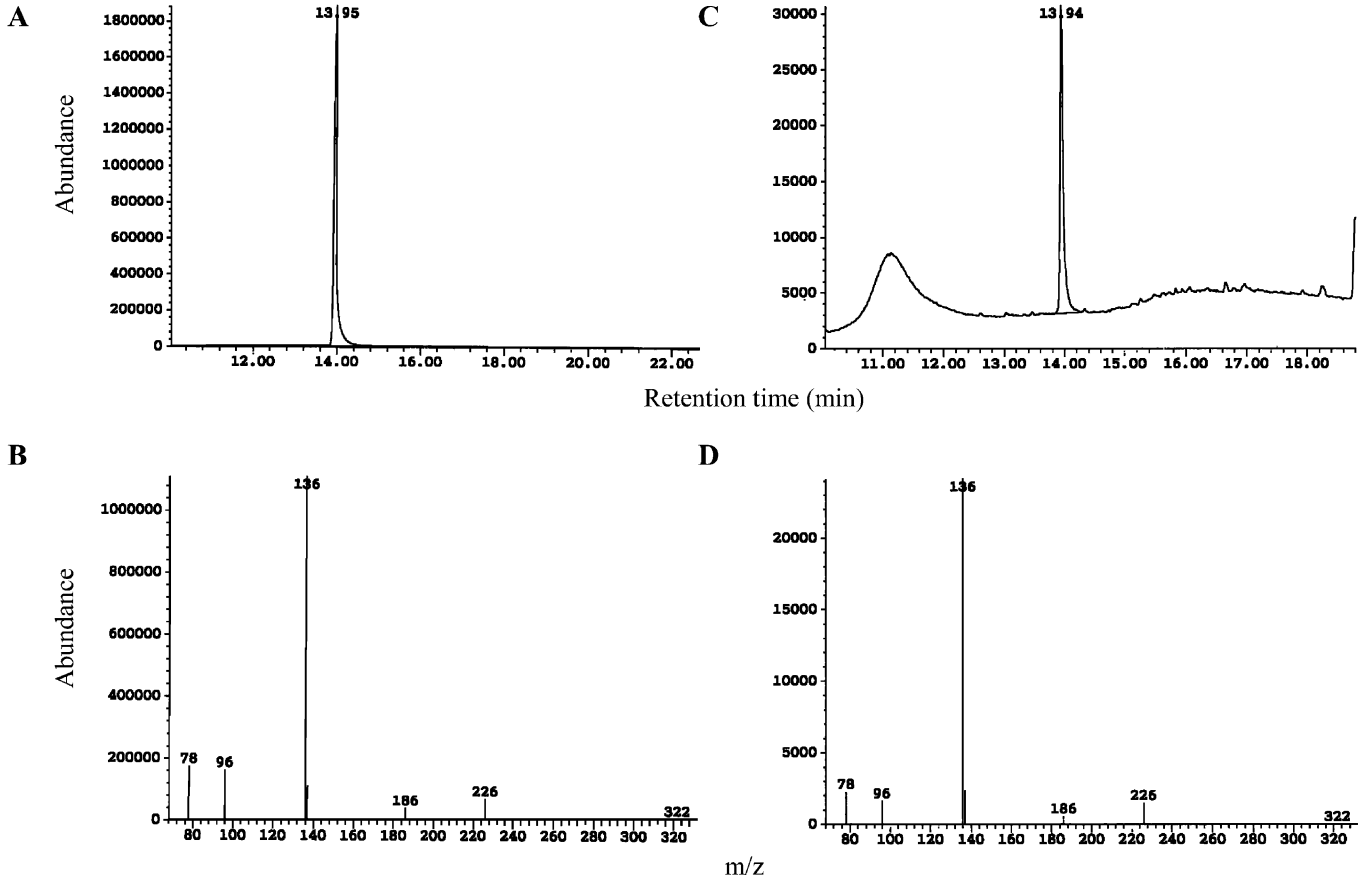

Fig. 3. Representative GC/MS confirming the presence of pyriproxyfen in treated *C. quadricarinatus* females. (A) GC-MS chromatogram of spiked pyriproxyfen std. serving as reference standard; (B) MS fragmentation of the peak in chromatogram A; (C) GC-MS chromatogram of pyriproxyfen, post treatment, isolated from the hepatopancreas of a treated female; (D) MS fragmentation of the peak in chromatogram C.

Table 2
Distribution of pyriproxyfen in different tissues of *C. quadricarinatus* females after 17 weeks of exposure to different pyriproxyfen levels and the concentrations of pyriproxyfen in the water of the treatment tanks at the end of the experiment

Treatment (μg/g animal body weight/week)	Muscle (μg/g)	Gills (μg/g)	Ovary (μg/g)	Hepatopancreas (μg/g)	Water (μg/ml)
Control (0)	ND	ND	ND	ND	ND
Pyriproxyfen I (0.2)	5.30 ± 1.13	7.30 ± 0.42	19.25 ± 3.89	95.50 ± 4.95	ND
Pyriproxyfen II (2)	0.72 ± 0.69	1.79 ± 0.36	10.33 ± 4.12	143.67 ± 34.53	0.01 ± 0.0
Pyriproxyfen III (20)	3.99 ± 1.07	78.5 ± 20.5	31.7 ± 6.65	561.00 ± 114.55	0.02 ± 0.0

ND—Not detected, below the limit of quantification.

Table 3
Summary of spawning, molting and survival in *C. quadricarinatus* females, and in females after 17 weeks of exposure to different doses of pyriproxyfen vs. control females and those that had undergone eyestalk ablation Different letters represent statistically significant difference (χ^2, $p < 0.05$) for each column.

Treatment (μg/g animal body weight/week)	Survival (%)	Spawning (%)	Molting (%)	Molt increment (% ± S.D.)
Control (0)	90.0	66.6[b]	44.0[b]	27.2 ± 5.4
Destalked (0)	80.0	93.7[a]	100.0[a]	30.4 ± 6.3
Pyriproxyfen I (0.2)	85.0	59.0[b]	52.9[b]	26.7 ± 8.0
Pyriproxyfen II (2)	80.0	43.7[b]	38.0[b]	20.2 ± 6.1
Pyriproxyfen III (20)	95.0	26.0[c]	63.1[b]	26.2 ± 6.1

levels showed lower, but not significantly different, rates of spawning vs. the control (Fig. 4, Table 3).

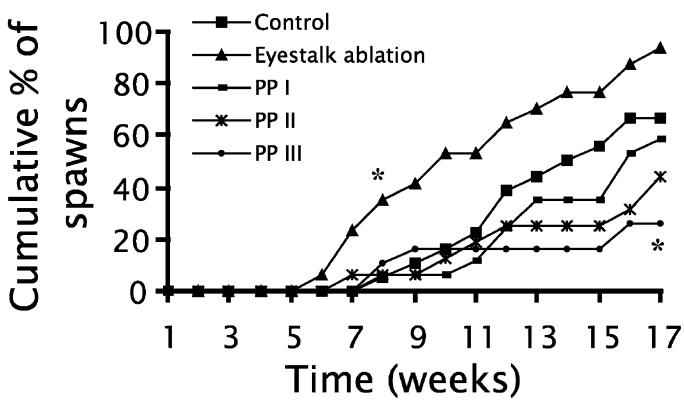

Fig. 4. Cumulative % of spawns in *C. quadricarinatus* females during 17 weeks of pyriproxyfen administration. PP I–0.2 μg pyriproxyfen/g animal body weight/week; PP II-2 μg pyriproxyfen/g animal body weight/week; PP III-20 μg pyriproxyfen/g animal body weight/week. Asterisks represent statistically significant difference ($p < 0.05$).

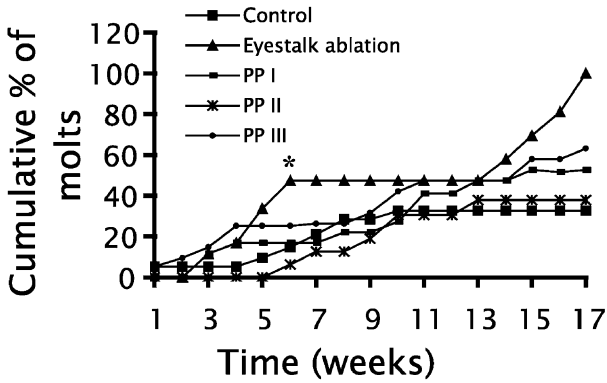

Fig. 5. Cumulative % of molts in *C. quadricarinatus* females during 17 weeks of pyriproxyfen administration. PP I–0.2 μg pyriproxyfen/g animal body weight/week; PP II-2 μg pyriproxyfen/g animal body weight/week; PP III-20 μg pyriproxyfen/g animal body weight/week. Asterisk represents a statistically significant difference ($p < 0.05$).

Cumulative % of molts for the group treated with the highest level of pyriproxyfen was somewhat higher than that for the control group from the 14th week onwards, and by the 17th week the difference became nearly significant (χ^2; $p = 0.065$). The cumulative molting events in the groups receiving the lowest and the intermediate levels of the pyriproxyfen were similar to those of the control (Fig. 5, Table 3). Molt increment (% of the weight increase from the premolt weight) was not affected by the different treatments, being approximately 26%, 27%, and 30% in the highest level of pyriproxyfen, the control and the destalked females, respectively (Table 3).

4. Discussion

MF is regarded as the predominant juvenile hormone-like compound in crustaceans (Laufer et al., 1987), and its presence has been shown in the hemolymph of a number of crustacean species (Homola and Chang, 1997b). The present study is the first report of MF in the hemolymph of the crayfish *C. quadricarinatus*.

Since the discovery of MF in crustaceans (Laufer et al., 1987), the function of this compound and its analogs in crustaceans has been subject to debate (Laufer et al., 1993; Homola and Chang, 1997b). Recent work in *P. clarkii* showed that exogenous MF can stimulate and enhance ovarian maturation by stimulating a great number of oocytes to mature (Laufer et al., 1998). However, the present study showed that administration of MF to mature *C. quadricarinatus* females did not seem to enhance spawning. Rather, it caused a tendency to molt acceleration and increased mortality rate. The juvenile hormone analog, pyriproxyfen, did not affect survival; however, it also caused a tendency to increase molting, and high doses of pyriproxyfen inhibited spawning. The discrepancies between the results of the current study and those of Laufer et al. (1998) may be due to differences in the reproductive physiology between *C. quadricarinatus*

and *P. clarkii* (Kulkarni et al., 1991; Abdu et al., 2000). Supporting evidence for this assumption comes from the differences in neuromuscular and ovarian neuroendocrine control between crayfish species from the southern hemisphere (family Parastacidae), such as *C. destructor*, and species from the northern hemisphere (family Cambaridae), such as *P. clarkii* (McRae and Mitchell, 1997). In addition, the effects of MF on gonad maturation was evaluated in *P. clarkii* in terms of the GSI (Laufer et al., 1998), whereas it was evaluated in terms of actual spawning in *C. quadricarinatus* in the present study.

In this study, continuous exposure of the female crayfish to pyriproxyfen led to its accumulation in a number of tissues, particularly, the hepatopancreas. This accumulation could be due to the absence of specific catalytic enzymes, such as esterases for MF (Homola and Chang, 1997a; Takac et al., 1997). However, this study tested the amount of pyriproxyfen in the tissues during chronic exposure, but did not address the issue of clearance of the compound with time after the end of the experiment. This calls for further investigation of the pharmokinetics of the administered pyriproxyfen, addressing the time span required for metabolism/excretion of the compound.

Pyriproxyfen has been reported to be acutely toxic to several crustacean species; for example, high doses of pyriproxyfen in the water caused mortality in shrimp (Brown et al., 1996) and in *Daphnia* (Trayler and Davis, 1996), and continuous exposure to lower doses suppressed both growth and reproduction in *Daphnia* (Trayler and Davis, 1996). In this study, we showed that orally administered MF had some toxic effects on *C. quadricarinatus*, whereas orally administered pyriproxyfen did not have lethal effects on reproductive females. The difference could be explained by the pharmacological effect of high levels of an endogenous hormone, in this case MF. A survey of different crustacean species showed that they have significant differences in their MF hemolymph titers, ranging from low levels in *Homarus americanus* (0.4–0.8 ng/ml) to high levels in *L. emarginata* (0.04–104 ng/ml) (Homola and Chang, 1997b). In this study, the level of MF in the treated animals was twice as high as that in the control. Thus, there is a need to test the effect of lower doses of MF.

The results of this study suggest that both compounds tested affect the balance between molt and reproduction. This finding could be explained in terms of the involvement of MF in the molt cycle (Homola and Chang, 1997b). Taking into account the possible involvement of MF and pyriproxyfen in molting and the antagonistic relationship between growth and reproduction in *C. quadricarinatus* (Barki et al., 1997), we suggest that MF and pyriproxyfen might induce molting and thereby inhibit its antagonistic process—spawning. To confirm this molt-inducing effect, future study on fast-growing crayfish individuals (i.e., young individuals and males) will be required.

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