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Methyl farnesoate couples environmental changes to testicular development in the crab *Carcinus maenas*

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SUMMARY

Carcinus maenas **males have two major color phases. Green-phase males molt frequently and tend to live in brackish estuaries during the summer. After becoming red-phase males, they molt infrequently, have higher mating success, and live in cooler, deeper water. We found profound differences between these two phases in the way salinity and temperature affect hemolymph levels of methyl farnesoate (MF), a hormone that affects crustacean reproduction. Few green-phase males (<10%) had detectable MF in 33 ppt seawater (SW) at 11 or 18°C. By contrast, about 30% of the red-phase males had detectable MF at either temperature. After transfer to 5 ppt SW, none of the green-phase males had detectable MF at 11°C whereas 100% of green-phase males did at 18°C. By contrast, 100% of the red-phase males had detectable MF in 5 ppt SW at either temperature. At 11°C, green-phase males had detectable MF after eyestalk ablation (ESA), showing that they can produce MF. There was no additional increase in MF levels when ESA animals of either color phase were transferred to 5 ppt SW, suggesting that the eyestalk is the primary regulator of the MF response to low salinity. MF levels of green-phase males were increased by injecting MF, by ESA, or by exposure to 5 ppt SW at 18°C. The testicular index of these treated animals nearly doubled after two weeks. Our results strongly suggest that environmental conditions such as temperature and salinity, affect testicular development in this crab by changing its MF levels.**

Key words: methyl farnesoate, testis, crustacean, salinity, reproduction, temperature.

INTRODUCTION

Environmental factors such as temperature, salinity and day length are known to affect crustacean reproduction (Robertson et al., 1991; Hoang et al., 2002; Carmona-Osalde et al., 2004). However, the hormonal mechanisms that mediate these environmental effects are largely unknown. It is well established that crustacean reproduction can be affected by eyestalk neuropeptides [e.g. gonad-stimulating and gonad-inhibiting hormones (Huberman, 2000; Borst, 2003; Ye et al., 2006)]. More recently, it has been shown that reproduction can be stimulated by methyl farnesoate (MF), a crustacean sesquiterpene related to the insect juvenile hormone. MF production is also regulated, in part, by eyestalk neuropeptides (Borst et al., 2001; Nagaraju, 2007). Finally, it has been shown that hemolymph levels of MF respond to environmental factors such as salinity and temperature (Lovett et al., 1997; Lovett et al., 2001). Thus, it seems plausible that MF is an endocrine link between the environment, eyestalk peptides and reproduction in crustaceans.

A useful model system for such studies is the green crab (also known as the shore crab) *Carcinus maenas*, an abundant crustacean found in rocky intertidal zones (Menge, 1995). This species was introduced to the East Coast of North America from Europe in the early nineteenth century and reached the West Coast approximately 20 years ago (Cohen et al., 1995). These euryhaline crabs can tolerate a wide range of salinities (1.4–52 ppt salinity) and temperatures (0–30°C) (Crothers, 1968; Cohen et al., 1995). Most importantly, many of the eyestalk neuropeptides in this species have been identified (Webster, 1998), and hemolymph levels of MF can be manipulated by salinity (Lovett et al., 2001).

C. maenas can have a range of colors on its ventral surface (e.g. light green, yellow, orange and red). Nevertheless, these crabs are usually divided into two groups: green-phase crabs and red-phase crabs (Kaiser et al., 1990; McKnight et al., 2000). As one of these colors is the green phase, we will refer to individuals of *C. maenas* hereafter simply as 'crabs' to avoid the cumbersome term 'green-phase green crabs'.

These color phases differ substantially in terms of their physiology and behavior. Green-phase animals tend to be smaller than red-phase animals and molt more frequently. However, after they become red-phase animals, they molt infrequently (Kaiser et al., 1990; McGaw et al., 1992; Reid et al., 1997; Wolf, 1998). Red-phase crabs are generally more sensitive to environmental stress than green-phase crabs; they extract oxygen from seawater less effectively and are less tolerant of hypoxia (Reid and Aldrich, 1989). Red-phase crabs are less effective osmoregulators and have higher mortality rates when exposed to low salinity seawater (Reid et al., 1989; McGaw and Naylor, 1992a; McGaw and Naylor, 1992b). However, red-phase animals are more robust (thicker carapace, larger chela muscles) and aggressive and have greater mating success than green-phase animals. These, and other observations, have led to suggestions that the two color phases represent a trade-off between molting and mating. Green-phase crabs put most of their resources into rapid growth but when they become red-phase crabs they put most of their resources into reproduction (Reid et al., 1997).

These physiological and behavioral differences affect the environments the crabs inhabit. Red-phase animals tend to live in deeper water along open shores where the water is cooler and is closer to full-strength seawater. By contrast, green-phase crabs migrate into inlets and estuaries during high tide during the summer (Crothers, 1968; Hunter and Naylor, 1993; Warman et al., 1993). Thus, green-phase animals are more likely to experience higher water temperatures and brackish water during the summer compared with red-phase crabs.

In the present study, we show that red-phase crabs are more likely to have detectable MF in their hemolymph than green-phase crabs. In addition, the testicular index (TI) of red-phase animals is higher than that in green-phase animals. Furthermore, our results indicate that environmental factors differentially affect MF levels in the two color phases. These results are consistent with the hypothesis that MF stimulates testicular development. To test this hypothesis, we show that environmental factors that increase MF levels also stimulate testicular development. The MF levels in these two color phases appear to be responsible, in part, for the different reproductive activities of these males.

MATERIALS AND METHODS

Animals

Crabs [*Carcinus maenas* (Linnaeus 1758)] were generously provided by Dan Landers (Millstone Environmental Lab; Waterford, CT, USA). Most of the males used in this study were 50±5 g (carapace width, CW 5.2±0.6 cm) and were collected during the spring and early summer. Green-phase and red-phase animals were identified by the color of their ventral surface (Reid et al., 1997). None of the experimental animals was missing appendages. At the end of some experiments, the testes were removed and weighed, and the TI (% total body mass) was calculated. Crabs were kept in large tanks containing artificial SW (Instant Ocean, Spectrum Brands; Atlanta, GA, USA) at normal salinity (33 ppt) at 11 or 18°C. Crabs were fed on squid and shrimp twice weekly. Animals were acclimated to the tanks for at least a week before being used in experiments.

Treatments

For some experiments, crabs were given a hyposalinity challenge by placing them in dilute SW (5 ppt) at 11 or 18°C for 24 h. MF levels were analyzed by taking hemolymph samples (0.5 ml) immediately prior to the transfer and 24 h later at the end of the challenge. Studies with [^{14}C]inulin indicate that the hemolymph volume of these animals ranges from 20 to 25% of their wet body mass (G.P.C.N. and D.W.B., unpublished data), hence 0.5 ml of hemolymph is approximately 4% of the crab's total hemolymph volume. Previous studies demonstrate that MF levels are not affected by this sampling frequency or volume (Lovett et al., 2001). Some animals were bilaterally eyestalk-ablated (ESA) by removing the eyestalks and then applying boric acid powder and Vaseline® to the wound. Animals were anesthetized on ice for at least 10 min prior to surgery or dissection. These animals were bled one day prior to ESA and then at the indicated times after ESA. Some animals were bled sequentially but never more frequently than 24 h.

Measurement of methyl farnesoate (MF) levels

Hemolymph levels of MF were determined using a high-performance liquid chromatography (HPLC) method that has been previously described and validated for *C. maenas* (Borst and Tsukimura, 1991). Briefly, hemolymph (0.5 ml unless otherwise noted) was withdrawn through the arthrodial membrane of the coxa of a walking leg. It was added to 2.5 ml acetonitrile and 4% NaCl to give a total volume of 4.5 ml. The mixture was extracted with 1.5 ml hexane containing 20 ng of *cis*,*trans*-MF (used as an internal standard) and the hexane supernatant was analyzed by normal-phase HPLC (silica column; 1.9% diethyl ether in hexane; 220 nm). We calculated the MF content of each sample by comparing its MF peak area with that of an MF standard. The lower limit of MF detection was 2 ng ml^{-1}.

Effect of salinity, temperature, ESA and MF treatment on testicular development

Male green-phase crabs were acclimated to 33 ppt SW at 11°C for at least one week. As stress often causes a small transient rise in MF levels (Lovett et al., 2001), the handling of these animals was limited and they were not bled. Some males were treated with 1 µg MF (Echelon Biosciences; Salt Lake, UT, USA). As MF is hydrophobic, we limited adsorption loss by suspending it, immediately prior to injection, in modified Pantin's saline (Laufer et al., 1987) containing 5% ethanol and 1% bovine serum albumin (BSA; Sigma-Aldrich Chemical Co., St Louis, MO, USA). Control animals were treated with the vehicle solution. MF and vehicle-treated animals were injected with 200 µl of the solution, through the arthrodial membrane of the coxa of the third walking leg, on days one, five and 10. The TI values of these animals were determined on day 15.

In a second experiment, males were treated with temperature, hyposalinity or ESA. Group 1 crabs (the initial control) were dissected on the first day of the experiment. Crabs in group 2 (intact) and group 3 (ESA) were maintained in 33 ppt SW at 11°C. Crabs in groups 4 and 5 were held in 18°C SW with a salinity of 33 ppt or 5 ppt, respectively. Animals from groups 2–5 were dissected on day 15, and their TI values were determined. No deaths or injuries occurred during the experiment.

Statistical analysis

The data were analyzed using Instat Software (GraphPad; San Diego, CA, USA). A two-tailed *t*-test was used to compare MF levels between the two different treatment groups. For data expressed as the percentage of a population, Fisher's exact test was used. Data with multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test to determine significance.

RESULTS

We measured MF levels in the hemolymph of green crabs maintained in 33 ppt SW at 11°C. As shown in Fig. 1A, the percentage of animals with detectable MF in their hemolymph (i.e. >2 ng ml^{-1}) was 8-fold greater in red-phase males than in green-phase males (P<0.01). The MF levels in these red-phase males with detectable MF were 23.8±3.1 ng ml^{-1} (N=12). The sole green-phase male with detectable MF had 18.6 ng ml^{-1}. We also compared the TI of red- and green-phase crabs. The TI of red-phase crabs was 79% greater than the TI of green-phase crabs (P<0.001; *t*-test; Fig. 1B).

The absence of detectable MF in the hemolymph of nearly all green-phase males suggests that these crabs might lack some part of the biochemical pathway needed to synthesize this sesquiterpene. We tested this hypothesis by comparing the effect of a hyposalinity challenge on MF levels of red-phase and green-phase crabs (Fig. 2), a treatment that chronically elevates hemolymph levels of MF in *C. maenas* (Lovett et al., 2001). In red-phase males, a hyposalinity challenge increased the percentage of animals with detectable MF from 37% to 94% at 11°C (P<0.001) and from 30% to 92% at 18°C (P<0.001) (Fig. 2A). Whereas low salinity increased the percentage of animals that had detectable levels of MF at either temperature, the amount of MF in the hemolymph of those animals with detectable MF did not significantly change (Fig. 2B).

By contrast, green-phase males showed little response to a hyposalinity challenge at 11°C (Fig. 2C). However, after acclimation to 33 ppt SW at 18°C for one week, green-phase males responded strongly to a hyposalinity challenge, and the percentage of animals

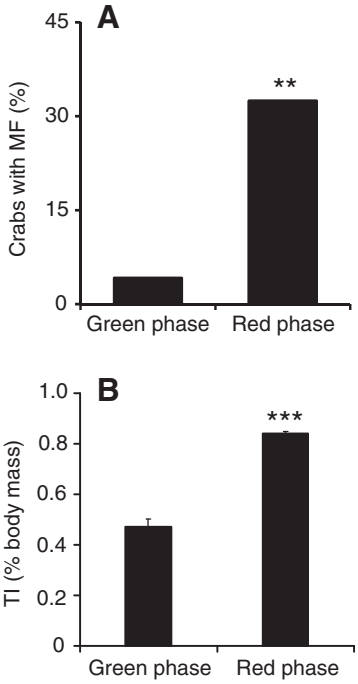

Fig. 1. Red-phase and green-phase male *Carcinus maenas* differ in the occurrence of methyl farnesoate (MF) in their hemolymph and in their testicular development. (A) Red-phase males (N=37) are more likely to have detectable MF (>2 ng ml^{-1}) in their hemolymph than green-phase animals (N=24; **P<0.01; Fisher's exact test). (B) The testicular index (TI) was 79% greater in red-phase than in green-phase crabs (values ± s.e.m.; N=12 for each group; ***P<0.001; Student's t-test).

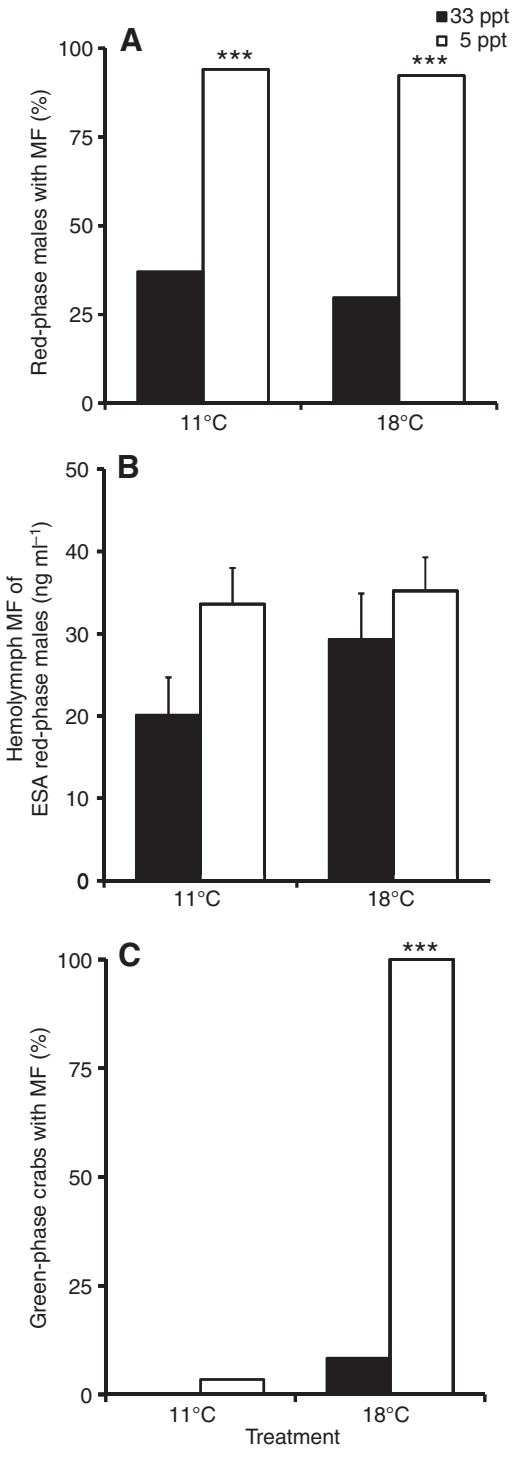

Fig. 2. Red-phase and green-phase male crabs respond differently to a hyposalinity challenge. Crabs were acclimated to 33 ppt seawater (SW) for one week and then transferred to 5 ppt SW for 24 h. (A) Some red-phase males (37 and 30%) had detectable MF in 33 ppt SW at 11°C and 18°C, respectively. Hyposalinity treatment increased the percentage (94 and 92%, respectively; N=12; ***P<0.001; Fisher's exact test). (B) Red-phase males with detectable MF had similar MF levels at each temperature and salinity (values ± s.e.m.; P>0.05; N=12). (C) Few green-phase crabs had detectable MF at either temperature in 33 ppt SW. Only one responded to 5 ppt SW at 11°C whereas 100% did at 18°C (N=12; ***P<0.001; Fisher's exact test).

with detectable MF increased from 8.3% to 100% (P<0.001). In other studies (not shown), we found that green-phase males acquired the ability to respond to dilute SW after acclimation to 18°C SW after only 24 h (the shortest period tested). When returned to SW at 11°C they lost the ability to respond. The MF levels (33±4.5 ng ml^{-1}) in green-phase males after a hyposalinity challenge at 18°C were not significantly different from the MF levels detected in red-phase males (35.2±4.1 ng ml^{-1}) under the same conditions.

Since acclimation to SW at 18°C might induce the enzymes of the MF synthetic pathway, we also investigated the effect of ESA on red- and green-phase males in 33 ppt SW at 11°C. Red-phase males were analyzed for MF approximately one week prior to ESA, and only those animals that had no detectable MF were included in this study. None of the animals in either color phase had detectable MF in hemolymph samples taken one day prior to ESA, whereas every animal in both groups had detectable MF 24 h after ESA. The MF levels of green-phase males (6.2±0.9 ng ml^{-1}, N=6) 24 h after ESA were significantly (P<0.001; t-test) lower than the levels observed in red-phase males (21.8±5.3 ng ml^{-1}, N=5). However, MF levels in green-phase males rose over time and on day 5 reached a level that was similar to the levels observed in red-phase males (Fig. 3A).

In a second experiment, we tested the effects of temperature and salinity on the MF levels of red- and green-phase males after ESA (Fig. 3B,C). Animals were tested at least five days after ESA to allow MF levels to stabilize. A hyposalinity challenge had no significant effect on the MF levels of green- or red-phase ESA males at either temperature, although both groups showed a modest increase (28 and 25%, respectively) to this treatment at 18°C. The

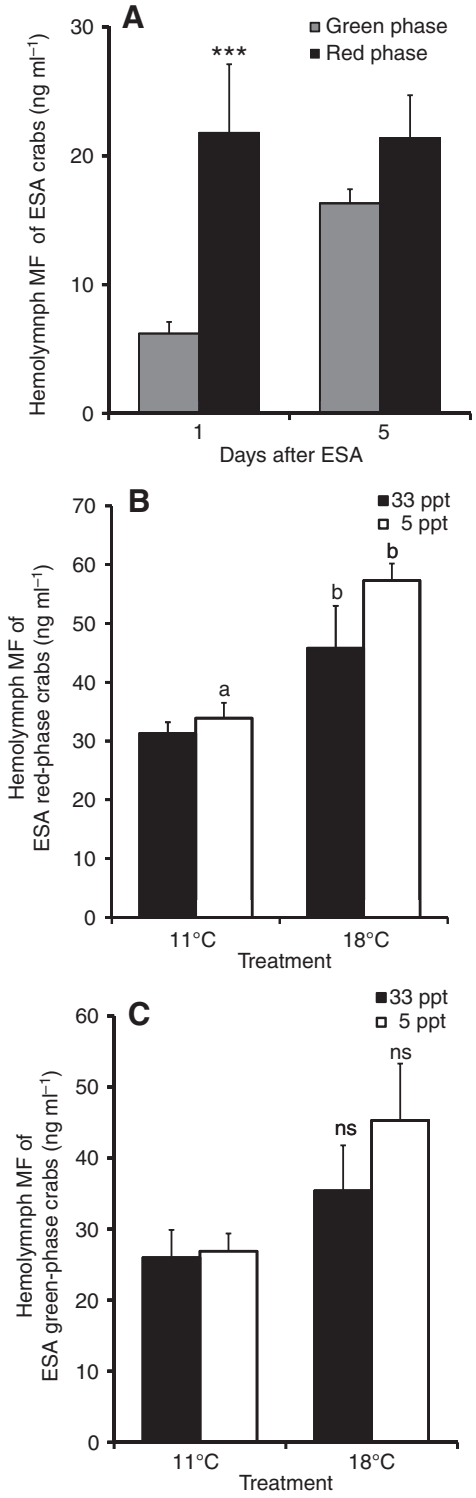

Fig. 3. Hemolymph levels of MF increase after eyestalk ablation (ESA). Animals without detectable methyl farnesoate (MF) were held in 33 ppt SW at 11°C until used. (A) All of the green-phase (N=6) and red-phase (N=5) males had detectable MF after ESA. On day 1, the amount of MF (values ± s.e.m.) was lower in green-phase males than in red-phase crabs (***P<0.001, t-test). By day 5 the levels were similar (P>0.05, t-test). (B) Red-phase (N=8) and (C) green-phase (N=7) males were treated with 5 or 33 ppt SW at 11 or 18°C, 5 or more days after ESA. SW at 5 ppt did not significantly increase MF levels of crabs in either color phase at either temperature. SW at 18°C increased MF levels, most notably at 5 ppt (bars with different letters = P<0.01, ns = P>0.05; ANOVA).

higher temperature significantly increased MF levels in red-phase males (Fig. 3B) and a similar trend was observed in green-phase males (Fig. 3C). This was most noticeable in 5 ppt SW, where the MF levels at 18°C were 68 and 69% higher in green- and red-phase animals, respectively.

The above data indicate that environmental factors (e.g. temperature and salinity) can affect MF production in *C. maenas*. We tested the potential impact of such factors on male reproduction. Green-phase males were initially housed in 33 ppt SW at 11°C. In one study, MF levels were increased by injecting males with 1 µg MF on days one, five and 10 while control animals were treated with the vehicle (Fig. 4A). By day 15, the TI of the MF-treated animals had increased by 119% (P<0.01; t-test). The TI of these treated animals was similar to the TI observed earlier in red-phase males (Fig. 1B).

We also determined the effects of other factors (e.g. ESA, hyposalinity treatment and temperature) on TI (Fig. 4B). The initial TI (day 0) and the TI of crabs held in 33 ppt SW at 11°C were 0.43 and 0.43, respectively. These values are similar to the TI of green-phase males observed earlier (Fig. 1B). ESA treatment significantly increased the TI by 90% (P<0.01). Transfer of crabs to 33 ppt SW at 18°C elevated the TI by 54% (P<0.01), whereas the transfer of crabs to 5 ppt SW at 18°C increased the TI by 103% (P<0.01). The TIs of the ESA males and those transferred to 5 ppt SW at 18°C were similar to those observed in red-phase males (Fig. 1B).

DISCUSSION

Our results extend previous studies of the two color phases of *C. maenas* and describe another physiological difference, the presence of MF in the hemolymph, between these two groups. More importantly, this study provides the first evidence that MF acts as a link between environmental changes and the stimulation of crustacean reproduction. Finally, our data indicate that the eyestalk plays a role in the increased MF levels observed in these crabs after transfer to dilute SW.

Red-phase males were much more likely to have MF in their hemolymph compared with green-phase animals. This observation correlates with the high TIs of red-phase animals, consistent with the idea that MF stimulates testicular growth either directly or indirectly in crustaceans (Kalavathy et al., 1999; Nagaraju et al., 2003; Nagaraju et al., 2006; Reddy et al., 2004). These observations are similar to previous studies in the spider crab (*Libinia emarginata*), which demonstrated a correlation between the reproductive status of males and MF synthesis by the mandibular organ (MO) and hemolymph levels of MF (Sagi et al., 1993; Sagi et al., 1994). It should be noted that the correlations between MF levels and male reproduction are weak, since the majority of the red-phase males do not show detectable MF but still have a high TI. The most likely explanation for this is that the spontaneous release of MF is episodic (Borst and Tsukimura, 1992) and the half-life of MF is short. In the lobster *Homarus americanus*, we estimated that the half-life of MF is less than 60 min (Tsukimura and Borst, 1992). However, a sustained elevation of MF does not appear to be necessary for a biological effect. MF treatments, such as those shown in Fig. 4A, would only cause a transient elevation of MF in these animals. Nevertheless, this treatment still affected testicular growth significantly.

Our data show that the two color phases differ profoundly in their responses to temperature and salinity. Previous studies showed that MF levels in *C. maenas* males increase when animals are exposed to dilute SW for more than 8 h (Lovett et al., 2001). In the Lovett et al. study, only green-phase animals were used because red-phase

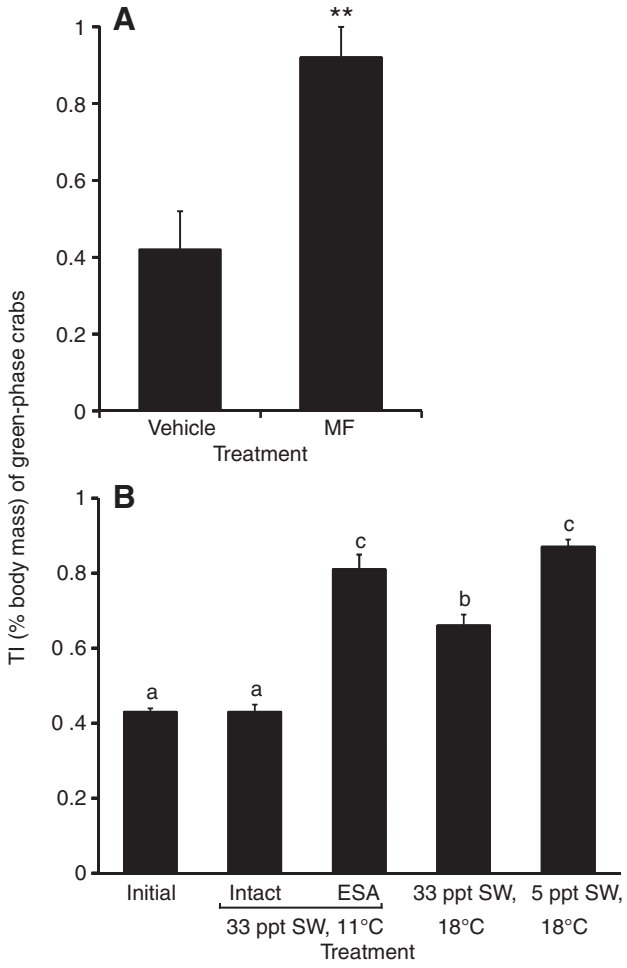

Fig. 4. The testicular index (TI) of green-phase males increased by treatments that elevate methyl farnesoate (MF) levels. Animals were treated for 15 days and the effect on their TI was determined. (A) Green-phase males were injected on days 1, 5, and 10 with either the vehicle (N=4) or with MF (N=6). MF treatment caused a significant increase in TI (**P<0.01). (B) Green-phase males were held in 33 ppt SW at 11°C prior to the experiment. One group of animals (N=6) was analyzed on the initial day of the experiment. Animals maintained in 33 ppt SW 11°C for 15 days (Intact, N=6) had no change in their TI whereas eyestalk ablation (ESA) animals (N=5) had a significantly elevation. Transfer of animals to 33 ppt SW at 18°C (N=6) for 15 days caused a moderate increase in TI, and transfer of animals to 5 ppt SW at 18°C (N=6) caused a larger increase in TI. Bars with different letters = P<0.05; ANOVA).

animals gave inconsistent results. These observations were confirmed by the current study. Untreated red-phase animals often have detectable MF, and the levels in these animals were similar to the levels observed in animals that responded to low salinity. Another significant difference between the two color phases is the temperature dependence of their response to a hyposalinity challenge. Red-phase males responded to a challenge at both temperatures (11 and 18°C) whereas green-phase males were only responsive at 18°C.

Eyestalk removal has been shown to increase MF levels in a wide variety of crustaceans, including *L. emarginata* (Laufer et al., 1987), *Cancer pagurus* (Borst et al., 2002), *Callinectes sapidus* (Henry and Borst, 2006) and *Oziotelphusa senex senex* (Nagaraju et al., 2005). Consistent with these observations, ESA increased MF levels in both green- and red-phase *C. maenas* males at 11°C. Clearly, green-phase animals can synthesize MF when held at 11°C. Thus, their lack of a response to hyposalinity at 11°C does not reflect an inability to produce MF. Nevertheless, the levels of MF observed in green-phase males 24 h after ESA were significantly lower than those observed in red-phase males. This suggests that the initial synthesis capacity of green-phase males is not as great as it is in red-phase males. The difference in MF levels between green- and red-phase animals disappears 5 days after ESA. This probably reflects the hypertrophy of the MO, which is known to occur after ESA (Borst et al., 1994; Nagaraju et al., 2004).

Although a hyposalinity challenge had no significant effect on the MF levels of green- or red-phase animals after ESA, there was a modest effect in SW at 18°C. This suggests that the eyestalk is the major regulator of MF levels in response to salinity. By contrast, temperature significantly affected the MF levels of ESA animals. It is unclear whether this is due to a temperature effect on overall metabolism or to a regulatory mechanism that is eyestalk independent.

Several compounds may regulate MF production by the MO, some of which are found in the eyestalk. Indeed, the simplest explanation for the elevation of MF levels of *C. maenas*, and other crustaceans, after ESA is that the eyestalk contains a compound that directly inhibits MF production by the MO. Several putative MO-inhibiting hormones (MOIHs) have been isolated from the sinus glands of *C. pagurus* (Wainwright et al., 1996) and *L. emarginata* (Liu and Laufer, 1996). Although these peptides can decrease MF synthesis by MO *in vitro*, they have not been shown to function as MOIHs *in vivo*. Indeed, relatively large amounts of both peptides are required to inhibit the MO *in vitro*, arguing against their role in regulating this gland *in vivo*. The sinus glands of *C. pagurus* and *H. americanus* contain another, as yet unidentified, peptide that inhibits MF production *in vivo* but does not appear to act directly on the MO (Borst et al., 2002). In addition, allatostatin has been shown to stimulate MF production by MO *in vitro* (Kwok et al., 2005), although it has not been shown to affect MF production *in vivo*. Clearly, more work needs to be done before we fully understand the mechanisms involved in regulating MF synthesis by this tissue.

Our present study provides strong evidence that MF has an important role in regulating male testicular development. A role for MF in male reproduction has been inferred from several previous studies. For example, the size of the MO in the male lobster (*H. americanus*) increases after sexual maturity (Waddy et al., 1995). Likewise, MF synthesis rates by spider crab MO incubated *in vitro* were correlated with reproductive behavior (Sagi et al., 1993; Sagi et al., 1994). In addition, MF treatment has been shown to increase testicular growth in a number of crustacean species, including the freshwater field crab *O. senex senex* (Kalavathy et al., 1999; Reddy et al., 2004), and the freshwater prawn *Macrobrachium malcholamsonii* (Nagaraju et al., 2003). Our observations confirm these previous studies and demonstrate that MF treatment can stimulate testicular growth in green-phase animals.

The eyestalk is also known to regulate testicular function in crustaceans. Ōtsu found that the removal of eyestalks from young male *Potama dehaani* resulted in a rapid increase in the size of the testes and male genital ducts (Ōtsu, 1961). Likewise, ESA of *Litopenaeus vannamei* increased their testicular index and doubled mating frequency (Chamberlain and Lawrence, 1981). These, and other, studies have led to the proposal that the eyestalk contains a gonad-inhibiting hormone (GIH) (Kulkarni et al., 1984; Ye et al., 2006). In the present investigation, we demonstrate that ESA stimulates testicular growth in green-phase males of *C. maenas*. As ESA increases MF levels, and MF treatment alone can stimulate testicular growth, it seems likely that MF couples ESA to gonad

growth in these animals. However, it should be noted that the green-phase males used in this study were large and were presumably nearing sexual maturity. If so, their responses to environmental conditions and other treatments may be different from the responses that would occur in younger (smaller) green-phase males. At younger stages, ESA may affect testicular development *via* mechanisms that do not include MF.

Finally, our present data show that environmental factors, such as temperature and salinity, can affect MF levels and thereby stimulate testicular growth. During the summer, green-phase animals are abundant in estuaries (Crothers, 1968; Hunter and Naylor, 1993; Warman et al., 1993), where the water is likely to be brackish and warmer. Thus, it seems likely that green-phase crabs will have a larger TI at the end of this season. Whether green-phase crabs seek an estuarine environment because it stimulates MF production and thereby increases gonad growth is not clear. Nevertheless, these observations provide a unique insight into how behavior, the environment and physiology might interact in the reproductive strategies of this crustacean. Determining these interactions will afford a more comprehensive view of how reproduction is regulated in this species and probably other crustaceans.

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REFERENCES

Borst, D. W. (2003). Crustacean endocrine systems. In *Encyclopedia of Hormones* (ed. H. L. Henry and A. W. Norman), pp. 340-351. Amsterdam: Elsevier.

Borst, D. W. and Tsukimura, B. (1991). Quantification of methyl farnesoate levels in hemolymph by high-performance liquid chromatography. *J. Chromatogr.* **545**, 71-78.

Borst, D. W. and Tsukimura, B. (1992). Methyl farnesoate levels in crustaceans. In *Insect Juvenile Hormone Research: Fundamental and Applied Approaches* (ed. B. Mauchamp, F. Couillaud and J. C. Baehr), pp. 27-35. Paris: Institut National de la Recherche Agronomique.

Borst, D. W., Tsukimura, B., Laufer, H. and Couch, E. F. (1994). Regional differences in methyl farnesoate production by the mandibular organ of the lobster, *Homarus americanus*. *Biol. Bull.* **186**, 9-16.

Borst, D. W., Ogan, J., Tsukimura, B., Claerhout, T. and Holford, K. C. (2001). Regulation of the crustacean mandibular organ. *Am. Zool.* **41**, 430-441.

Borst, D. W., Wainwright, G. and Rees, H. H. (2002). *In vivo* regulation of the mandibular organ in the edible crab, *Cancer pagurus*. *Proc. R. Soc. Lond. B Biol. Sci.* **269**, 483-490.

Carmona-Osalde, C., Rodríguez-Serna, M., Olvera-Novoa, M. A. and Gutieírrez-Yurrita, P. J. (2004). Effect of density and sex ratio on gonad development and spawning in the crayfish *Procambarus ilamasi*. *Aquaculture* **236**, 331-339.

Chamberlain, G. W. and Lawrence, A. L. (1981). Effect of light intensity and male and female eyestalk ablation on reproduction of *Penaeus stylirostris* and *P. vannamei*. *J. World Aquac. Soc.* **12**, 357-372.

Cohen, A. D., Carlton, J. T. and Fountain, M. C. (1995). Introduction, dispersal and potential impacts of the green crab *Carcinus maenas* in San Francisco Bay, California. *Mar. Biol.* **122**, 225-237.

Crothers, J. H. (1968). The biology of the shore crab, *Carcinus maenas* (L.). 2. The life of the adult crab. *Field Studies* **2**, 579-614.

Henry, R. P. and Borst, D. W. (2006). Effects of eyestalk ablation on carbonic anhydrase activity in the euryhaline blue crab *Callinectes sapidus*: neuroendocrine control of enzyme expression. *J. Exp. Zool.* **305**, 23-31.

Hoang, T., Lee, S. Y., Keenan, C. P. and Marsden, G. E. (2002). Effect of temperature on spawning of *Penaeus merguiensis*. *J. Therm. Biol.* **27**, 433-437.

Huberman, A. (2000). Shrimp endocrinology. A review. *Aquaculture* **191**, 191-208.

Hunter, E. and Naylor, E. (1993). Intertidal migration by the shore crab *Carcinus maenas*. *Mar. Ecol. Prog. Ser.* **101**, 131-138.

Kaiser, M. J., Hughes, R. N. and Reid, D. G. (1990). Chelae morphometry, prey-size selection and aggressive competition in green and red forms of *Carcinus maenas* (L.). *J. Exp. Mar. Biol. Ecol.* **140**, 121-134.

Kalavathy, Y., Mamatha, P. and Reddy, P. S. (1999). Methyl farnesoate stimulates testicular growth in the freshwater crab *Oziotelphusa senex senex* Fabricius. *Naturewissenschaften* **86**, 394-395.

Kulkarni, G. K., Nagabhushanam, R. and Joshi, P. K. (1984). Neuroendocrine control of reproduction in the male penaeid prawn, *Parapenaeopsis hardwickii* (Miers) (Crustacea, Decapoda, Penaeidae). *Hydrobiologia* **108**, 281-289.

Kwok, R., Zhang, J. R. and Tobe, S. S. (2005). Regulation of methyl farnesoate production by mandibular organs in the crayfish, *Procambarus clarkii*; a possible role for allatostatins. *J. Insect Physiol.* **51**, 367-378.

Laufer, H., Borst, D. W., Baker, F. C., Carrasco, C., Sinkus, M., Reuter, C. C., Tsai, L. W. and Schooley, D. A. (1987). Identification of a juvenile hormone-like compound in a crustacean. *Science* **235**, 202-205.

Liu, L. and Laufer, H. (1996). Isolation and characterization of sinus gland neuropeptides with both mandibular inhibiting hormone and hyperglycemic effects from the spider crab, *Libinia emarginata*. *Arch. Insect. Biochem. Physiol.* **32**, 375-385.

Lovett, D. L., Clifford, P. D. and Borst, D. W. (1997). Physiological stress elevates hemolymph levels of methyl farnesoate in the green crab *Carcinus maenas*. *Biol. Bull.* **193**, 266-267.

Lovett, D. L., Verzi, M. P., Clifford, P. D. and Borst, D. W. (2001). Hemolymph levels of methyl farnesoate increase in response to osmotic stress in the green crab *Carcinus maenas*. *Comp. Biochem. Physiol.* **128**, 299-306.

McGaw, I. J. and Naylor, E. (1992a). Salinity preference of the shore crab *Carcinus maenas* in relation to coloration during inter-moult and to prior acclimation. *J. Exp. Mar. Biol. Ecol.* **155**, 145-159.

McGaw, I. J. and Naylor, E. (1992b). Distribution and rhythmic locomotor patterns of estuarine and open-shore populations of *Carcinus maenas*. *J. Mar. Biolog. Assoc. UK* **72**, 599-609.

McGaw, I. J., Kaiser, M. J., Naylor, E. and Hughes, R. N. (1992). Intraspecific morphological variation related to the moult-cycle in colour forms of the shore crab *Carcinus maenas*. *J. Zool. (Lond.)* **228**, 351-359.

McKnight, A., Mathews, L. M., Avery, R. and Lee, K. T. (2000). Distribution is correlated with color phase in green crabs, *Carcinus maenas* (Linnaeus, 1758) in southern New England. *Crustaceana* **73**, 643-770.

Menge, B. A. (1995). Indirect effects in marine rocky intertidal interaction webs: Patterns and importance. *Ecol. Monogr.* **65**, 21-74.

Nagaraju, G. P. C. (2007). Is methyl farnesoate a crustacean hormone? *Aquaculture* **272**, 39-54.

Nagaraju, G. P. C., Suraj, N. J. and Reddy, P. S. (2003). Methyl farnesoate stimulates gonad development in *Macrobrachium malcolmsonii* Milne Edwards. *Crustaceana* **76**, 1171-1178.

Nagaraju, G. P. C., Reddy, P. R. and Reddy, P. S. (2004). Mandibular organ, its relation to body weight sex molt and reproduction in the crab *Oziotelphusa senex senex* Fabricius (1791). *Aquaculture* **232**, 603-612.

Nagaraju, G. P. C., Prasad, G. L. V. and Reddy, P. S. (2005). Isolation and characterization of mandibular organ-inhibiting hormone from the eyestalks of freshwater crab, *Oziotelphusa senex senex*. *Int. J. Appl. Sci. Eng.* **3**, 61-68.

Nagaraju, G. P. C., Reddy, P. R. and Reddy, P. S. (2006). *In vitro* methyl farnesoate secretion by mandibular organs isolated from different molt and reproductive stages of the crab Oziotelphusa senex senex. *Fish. Sci.* **72**, 410-414.

Ōtsu, T. (1961). The accelerating influence of eyestalk ablation on spermatogenesis and on development of the male genital ducts in young *Potamon dehaani*. *Bull. Yamagata Univ. Ser. Nat. Sci.* **5**, 379-383.

Reddy, P. R., Nagaraju, G. P. C. and Reddy, P. S. (2004). Involvement of methyl farnesoate in the regulation of molting and reproduction in the freshwater crab *Oziotelphusa senex senex*. *J. Crust. Biol.* **24**, 511-515.

Reid, D. G. and Aldrich, J. C. (1989). Variation in responses to environmental hypoxia of different colour forms of the shore crab *Carcinus maenas* (L.). *Comp. Biochem. Physiol.* **92**, 535-539.

Reid, D. G., Abelló, P., McGaw, I. and Naylor, E. (1989). Differential tolerances of desiccation and hypo osmotic stress in sub- and inter-tidal *Carcinus maenas*. In *Phenotypic Responses in Aquatic Ectotherms* (ed. J. C. Aldrich), pp. 89-96. Japaga: Dublin.

Reid, D. G., Abelló, P., Kaiser, M. J. and Warman, C. G. (1997). Carapace colour, inter-moult duration and the behavioural and physiological ecology of the shore crab *Carcinus maenas*. *Estuar. Coast. Shelf Sci.* **44**, 203-211.

Robertson, L., Bray, W. and Lawrence, A. (1991). Reproductive response of *Penaeus stylirostris* to temperature manipulation. *J. World Aquac. Soc.* **22**, 109-117.

Sagi, A., Homola, E. and Laufer, H. (1993). Distinct reproductive types of male spider crabs *Libinia emarginata* differ in circulating and synthesizing methyl farnesoate. *Biol. Bull.* **185**, 168-173.

Sagi, A., Ahl, J. S. B., Danaee, H. and Laufer, H. (1994). Methyl farnesoate levels in male spider crabs exhibiting active reproductive behavior. *Horm. Behav.* **28**, 261-272.

Tsukimura, B. and Borst, D. W. (1992). Regulation of methyl farnesoate in the hemolymph and mandibular organ of the lobster, *Homarus americanus*. *Gen. Comp. Endocrinol.* **86**, 297-303.

Waddy, S. L., Aiken, D. E. and de Kleijn, D. P. V. (1995). Control of growth and reproduction. In *Biology of the Lobster Homarus americanus* (ed. J. R. Factor), pp. 217-266. San Diego: Academic Press.

Wainwright, G., Webster, S. G., Wilkinson, M. C., Chung, J. S. and Rees, H. H. (1996). Structure and significance of mandibular organ-inhibiting hormone in the crab *Cancer pagurus*. *J. Biol. Chem.* **271**, 12749-12754.

Warman, C. G., Reid, D. G. and Naylor, E. (1993). Variation in the tidal migratory behaviour and rhythmic light-responsiveness in the shore crab, *Carcinus maenas*. J. Mar. Biol. Assoc. UK **73**, 355-364.

Webster, S. G. (1998). Neuropeptides inhibiting growth and reproduction in crustaceans. In *Recent Advances in Arthropod Endocrinology* (ed. G. M. Coast and S. G. Webster), pp. 33-52. Cambridge: Cambridge University Press.

Wolf, F. (1998). Red and green colour forms in the common shore crab *Carcinus maenas* (L.) (Crustacea: Brachyura: Portunidae): Theoretical predictions and empirical data. *J. Nat. Hist.* **32**, 1807-1812.

Ye, H., Huang, H., Li, S., Wang, G. and Li, Q. (2006). *In vitro* study of neuroendocrine regulation over the testicular development in mud crabs *Scylla serrata*. *Chinese J. Oceanol. Limnol.* **24**, 142-146.