In vivo regulation of the mandibular organ in the edible crab, *Cancer pagurus*

D. W. Borst^{1*}, G. Wainwright² and H. H. Rees²

¹Department of Biological Sciences, Illinois State University, Normal, IL 61790-4120, USA

²School of Biological Sciences, University of Liverpool, Liverpool L69 7ZB, UK

Considerable evidence indicates that methyl farnesoate (MF) production by the crustacean mandibular organs is negatively regulated by neuropeptides from the sinus gland (SG) in the eyestalk. In the crab *Cancer pagurus*, two neuropeptides (MO-IH-1 and -2) have been isolated from the SG that inhibit MF synthesis by mandibular organs of female crabs *in vitro*. To test their activity *in vivo*, we treated eyestalk-ablated male crabs with SG extracts (SGEs) or MO-IH-1 and -2. SGEs reduced haemolymph levels of MF by 60–80%, while MO-IH-1 and -2 had little effect. Protease treatment of SGEs destroyed the *in vivo* activity, suggesting that the extract contains an additional peptide responsible for the *in vivo* activity. When separated by reversed-phase high performance liquid chromatography (HPLC), the *in vivo* activity eluted in fractions prior to MO-IH-1 and -2. When mandibular organs were removed from animals previously treated *in vivo* with these active fractions, they had reduced levels of MF synthesis and activity of farnesoic acid *O*-methyl transferase compared with mandibular organs from animals treated with saline. Together, these results indicate that the regulation of the crustacean mandibular organ is complex and may involve several SG compounds. Some of these compounds (i.e., MO-IH-1 and -2) act directly on the tissue while others affect the mandibular organ indirectly.

Keywords: mandibular organ; methyl farnesoate; mandibular organ-inhibiting hormone (MO-IH)

1. INTRODUCTION

A major product of the crustacean mandibular organ is the sesquiterpene methyl farnesoate (MF), a compound that is structurally similar to the insect juvenile hormones (JH) (Borst *et al.* 1987; Laufer *et al.* 1987a). The importance of JH in the regulation of insect metamorphosis, reproduction and behaviour (for reviews, see Riddiford 1994; Wyatt & Davey 1996; Gäde *et al.* 1997) has led to the hypothesis that MF may have similar functions in crustaceans (Borst & Laufer 1990). While the role of MF in crustaceans remains somewhat inconclusive (Homola & Chang 1997), recent studies suggest that MF may regulate reproduction and metamorphosis (Yamamoto *et al.* 1997; Abdu *et al.* 1998; Reddy & Ramamurthi 1998; Laufer *et al.* 1998; Smith *et al.* 2000).

One factor that has hindered physiological studies of MF is the location of the mandibular organ, which makes it difficult to remove surgically. As a result, it has been difficult to carry out 'classical' ablation–replacement studies to investigate MF function. Likewise, the rapid degradation of MF (estimated half-life in lobsters is *ca.* 30 min) and the episodic nature of its release (Borst & Tsukimura 1992) have made treatment protocols difficult.

An alternative approach for such studies would be to treat animals with compounds that can either inhibit or stimulate MF production by the mandibular organ. Several studies have demonstrated that MF synthesis is inhibited by a factor from the eyestalk (Laufer *et al.* 1987b; Tsukimura & Borst 1992). Hence, eyestalk ablation causes a rise in the haemolymph levels of MF (Laufer *et al.* 1987a; Tsukimura & Borst 1992) as well as the rate of

MF synthesis by the mandibular organ *in vitro* (Laufer *et al.* 1987b; Wainwright *et al.* 1996). Treatment of eyestalk ablated (ESA) animals with sinus gland (SG; a neurohaemal organ in the eyestalk) extract causes a rapid, dose-dependent decrease in MF levels (Tsukimura & Borst 1992). Similarly, mandibular organs incubated *in vitro* with SGEs synthesized less MF when compared with mandibular organs incubated alone (Laufer *et al.* 1987b; Wainwright *et al.* 1996).

These observations led to the isolation of several SG peptides that inhibit MF production by the mandibular organ (mandibular organ inhibiting hormone; MO-IH). In one study, two related peptides (MO-IH-1 and MO-IH-2), each containing 78 residues, were isolated and sequenced from the SG of *Cancer pagurus* (Wainwright *et al.* 1996). Of particular interest was the observation that mandibular organ responsiveness to these peptides changed during the vitellogenic cycle, being maximal at the beginning of vitellogenesis. In another study, three peaks of bioactivity were separated by reversed-phase HPLC from the SG of *Libinia emarginata*, one of which has been sequenced and contains 72 amino acids (Liu & Laufer 1996; Liu *et al.* 1997). In both of these studies, the active peptides were identified using *in vitro* bioassays.

The MO-IH peptides from both of these species belong to the CHH/MIH/VIH family of crustacean peptides (Keller 1992). The CHH (crustacean hyperglycemic hormone) subfamily contains peptides with 72–73 amino acids and a blocked N-terminus. The MIH (moult-inhibiting hormone)/VIH (vitellogenesis-inhibiting hormone) subfamily contains peptides with 77–78 amino acids and a free N-terminus. The mature peptides from different species within each subfamily have similar amino-acid sequences (greater than 50% identity). However, the amino-acid sequences of peptides in one

*Author for correspondence (dwborst@ilstu.edu).

subfamily have much lower similarity (*ca.* 19%) to those in the other subfamily (Keller 1992). Interestingly, biological function does not appear to be strictly related to a peptide's membership of a specific subfamily. Thus, the peptide with moult-inhibiting activity in *Carcinus maenas* belongs to the MIH subfamily while this peptide in *Homarus americanus* belongs to the CHH subfamily. An analogous situation appears to exist for the MO-IH peptides. The two MO-IH peptides isolated from *C. pagurus* are more similar to the MIH subfamily and the MO-IH form has been shown to have a hyperglycaemic effect *in vivo* (Liu & Laufer 1996), consistent with its membership of the CHH subfamily. While none of these peptides have been tested for their effects on MF levels *in vivo*, their suppression of MF synthesis by mandibular organs *in vitro* strongly supports the assumption that these peptides have a biological function *in vivo*.

We directly tested this assumption in *C. pagurus* and show that physiological doses of both MO-IH peptides from this species have no effect *in vivo* on haemolymph levels of MF under the conditions used. By contrast, SGEs do depress MF levels *in vivo*, an effect that appears to reflect the presence of another factor in the SG. This peptide regulates mandibular organ function *in vivo* but has no effect when incubated with this tissue *in vitro*, suggesting that either it acts indirectly upon the mandibular organ or that it must be activated *in vivo* before it can affect this tissue.

2. MATERIAL AND METHODS

(a) Animals

Crabs (*C. pagurus*) were obtained from fishermen in North Wales and maintained in Liverpool in large recirculating tanks of fresh seawater (18–20 °C) collected locally. Animals were fed once or twice per week with squid. Crabs (*ca.* 300 g) used in the bioassay were eyestalk ablated and marked so that individual animals could be identified.

(b) Preparation of SGEs and peptides

SG peptides were fractionated by reversed-phase HPLC as previously described (Wainwright *et al.* 1996). SG tissue was dissected from eyestalks and stored frozen at –80 °C. At the time of analysis, the SG tissue was sonicated in 2 M acetic acid and then centrifuged (14 000 *g* for 5 min). For some studies, this SGE was used directly. In other cases, the SGE was fractionated using a μ BondaPak phenyl column (60 Å, 10 μ ; 300 $\times$ 4.6 mm) reversed-phase column (Waters), with a linear gradient of acetonitrile: water (18–48%, 0.5% min^{-1} ; 1 ml min^{-1} ; 214 nm) containing 0.1% trifluoroacetic acid. The eluted fractions were collected in Minisorb tubes (Nunc) and stored at 4 °C either in solution after adding 0.1% methylthioethanol or after drying under vacuum. Following localization of the material exhibiting *in vivo* MO-IH activity on an HPLC chromatogram (see figure 5), HPLC fractions corresponding to this same region were obtained from a bulk extract of 500 SG (generously provided by Dr J. S. Chung and Dr S. G. Webster, University of Wales, Bangor). For some studies, aliquots of SGE (1.5 SG) were dried, resuspended in 500 μ l of 0.4 M ammonium bicarbonate (pH 7.9) with or without enzyme (10 μ g of trypsin, Roche; or 10 μ g proteinase K, Qiagen) and incubated for 1 h at 37 °C. Other aliquots were boiled for 2 min. These were then placed on ice until analysed in the bioassay.

Figure 1. SGEs caused a time- and dose-dependent decrease in haemolymph levels of MF. ESA adult male crabs were treated with SGE. Haemolymph samples were obtained prior to, and after, treatment and their MF levels determined. MF levels after treatment are expressed as the percentage of the initial MF levels. (a) Time-course of the effect: MF levels were measured at the indicated times after treatment with one equivalent of SGE (* $p < 0.05$; ** $p < 0.01$; *t*-test, $n = 3$ for each point). Filled squares represent SGE treated animals and filled triangles represent control. (b) Dose-response effect: animals ($n = 4$ –5 per treatment except 0.03 and 1 SG where $n = 2$) were treated with the indicated amounts of SGE and the MF levels determined at 3 h. The results were analysed by ANOVA; means with the same letter are not significantly different ($p > 0.05$). ($F_{5,17} = 14.01$, $p = 0.0001$).

(c) *In vivo* MO-IH bioassay

Animals used in the bioassay were ESA to remove endogenous sources of MO-IH and to increase haemolymph levels of MF. At least two days after eyestalk ablation, animals were injected at the base of the third walking leg with an aliquot of SGE or an HPLC fraction of the SGE. The SGEs or HPLC fractions were resuspended in crustacean saline (Webster 1986) sup-

plemented with 0.1% bovine serum albumin. Since haemolymph levels of MF return to initial levels within 24 h, these animals could be reused for subsequent bioassays.

(d) Measurement of MF

Haemolymph levels of MF were measured by HPLC essentially as described by Borst & Tsukimura (1991). One ml of haemolymph was removed from a walking leg and placed in a culture tube containing 2 ml of acetonitrile, 1 ml of 4% (w/v) NaCl, and 20 ng of *cis,trans*-MF (used as an internal standard). After chilling the tubes, 1 ml of hexane was added and the tubes vortexed and centrifuged (1000 *g* for 5 min). An aliquot of the upper phase in each tube was removed and 400 µl were analysed for MF content using normal-phase HPLC (0.9% (v/v) diethyl ether in hexane, 4 ml min⁻¹; 214 nm). The MF content of each sample was calculated by comparing the area of its MF peak with that of a known amount of MF standard as well as by comparison with the peak area of the internal standard. Peak area integration was carried out using UNIPPOINT software (Gilson, France). These calculations gave comparable results.

Haemolymph levels of MF in intact and ESA animals can differ substantially, so haemolymph samples were taken immediately before treatment (*T* = 0 h) and then several hours after treatment (unless otherwise noted, *T* = 3 h). The response of the mandibular organ to treatment was calculated by normalizing the MF levels of the treated animals to their initial MF levels ($MF_{t=3}/MF_{initial} \times 100$ = per cent initial MF level). In some cases, this value was subtracted from 100 to determine the inhibition index. Occasionally, a sample yielded a negative inhibition index, which was set to zero if reanalysis did not confirm the negative response.

(e) In vitro MO-IH bioassay

Peptide fractions or total SGEs were tested for activity as previously described (Wainwright *et al.* 1996). Briefly, each fraction was dried and resuspended in M199 culture medium supplemented with crustacean salts (Brody & Chang, 1989). MF synthesis was determined by incubating mandibular organs for 24 h in culture medium supplemented with 37 kBq [³H-methyl]-*L*-methionine (Amersham Pharmacia Biotech, UK; 2.92 TBq mmol⁻¹). Following incubation, tissue was homogenized in 4% (w/v) NaCl in water and acetonitrile (2:5 v/v; 700 µl). The radiolabelled products were extracted by partition into hexane (500 µl) and analysed by reversed-phase HPLC with a A500 series FloOne online radioactivity detector (Canberra Packard).

(f) Radioimmunoassay of MO-IH

Radioimmunoassay of *C. pagurus* MO-IHs was carried out, essentially, as previously described for CHH (Webster 1996). MO-IH-1 was purified from SG of *C. pagurus* by HPLC (Wainwright *et al.* 1996). For the initial immunization, rabbits were treated with 1 nmol of MO-IH-1 suspended in Freund's complete adjuvant. The same amount of antigen was suspended in Freund's incomplete adjuvant for each of the booster injections.

The radioligand was prepared by iodinating MO-IH-1 (300 pmol) using lactoperoxidase immobilized on Sephadex beads (a gift of Dr Simon Webster, University of Wales, Bangor), carrier-free Na¹²⁵I (11.1 MBq; Amersham Pharmacia Biotech, UK) and 0.5 nM hydrogen peroxide. After 5 min, the reaction was quenched by adding excess KI and the mixture applied to a PD-10 column (Sephadex G25; Amersham Pharm-

acia Biotech, UK) and eluted with 50 mM phosphate buffer (pH 7.2). Fractions containing radioiodinated MO-IH were combined and used for the radioimmunoassay.

For the radioimmunoassay, a standard curve was prepared containing known quantities of *C. pagurus* MO-IH-1 ranging from 0 to 1000 fmol in 100 µl RIA buffer (50 mM phosphate buffer, pH 7.2, containing 0.1% (w/v) bovine serum albumin). Samples to be tested were dried and resuspended in 100 µl of RIA buffer. Thereafter, 100 µl aliquots of radioiodinated MO-IH-1 (*ca.* 6000 cpm in RIA buffer) and anti-MO-IH-1 antiserum (diluted 1:2000 in 100 µl of RIA buffer to give a maximum binding of 25% at a final dilution of 1:6000) were added to standard or sample tubes. The tubes were incubated for 18 h at 4 °C. Bound and free [¹²⁵I]MO-IH were separated by incubating each tube with 50 µl of goat anti-rabbit IgG immobilized on cellulose particles (SacCell; Immunodiagnostic Services Ltd) for 30 min at room temperature. After centrifugation (1000 *g* for 1 min), the supernatant was aspirated from each tube and the pellet was resuspended in 1 ml of 50 mM phosphate buffer (pH 7.2). This was centrifuged (1000 *g* for 1 min) again, the supernatant aspirated and the pellet assayed for radioactivity. The standard curve was used to estimate the amount of MO-IH-1-like immunoreactivity in each sample. The limit of detection of this assay was estimated to be *ca.* 6 fmol MO-IH-1 per tube. MO-IH-2 exhibited a marginally (6%) better cross-reaction with the antiserum compared with MO-IH-1.

(g) MeT assay

The activity of farnesoic acid *O*-methyl transferase (MeT) was measured as previously described (Wainwright *et al.* 1998). Briefly, post-microsomal supernatants (cytosol) prepared from mandibular organs were incubated for 30 min at 37 °C in the presence of 2.4 µM [³H]farnesoic acid (0.10 GBq mmol⁻¹; greater than 99% purity by HPLC) and 30 µM *S*-adenosyl-*L*-methionine with a final reaction volume of 50 µl. [³H]Farnesoic acid was prepared by hydrolysis of [³H]MF (the gift of Dr Glenn Prestwich, University of Utah) as described previously (Wainwright *et al.* 1998). Reactions were terminated by adding 150 µl of CH₃CN. After centrifugation (10 000 *g* for 10 min), a portion of the supernatant (usually 120 µl) was analysed by reversed-phase HPLC using online radioactivity monitoring as described in § 2(f). The conversion of [³H]farnesoic acid to [³H]MF in each sample was calculated. Results are expressed as pmol MF produced per 30 min per 100 µg of cytosol protein.

3. RESULTS

Male *C. pagurus* were eyestalk ablated to remove the only known endogenous source of MO-IH peptides. As expected, ESA animals had higher haemolymph levels of MF (45 ± 20 ng ml⁻¹, *n* = 5) than intact males (22 ± 13 ng ml⁻¹, *n* = 5) one day after the operation. While this apparent twofold increase was not statistically significant (*p* > 0.05, *t*-test) due to the variability observed in both intact and ESA animals, it is similar in magnitude to the difference in MF synthesis observed in mandibular organs from intact and ESA female crabs (Wainwright *et al.* 1996). When ESA animals were treated with SGE, a time-dependent decrease in haemolymph levels of MF was observed (figure 1a), with the maximum response observed after 3 h. Therefore, in subsequent bioassays, animals were tested 3 h after treatment. The effect of the SG extract was dose-dependent, with a 50% response (ED₅₀) of *ca.* 0.1 SG (figure 1b).

Figure 2. Purified MO-IH-1 and -2 had no activity *in vivo*. ESA males were treated as in figure 1b with saline ($n = 5$), the extract of 0.5 SG (SGE; $n = 7$) or 20 pmol of purified MO-IH-1 ($n = 6$) or MO-IH-2 ($n = 3$). The results were analysed by ANOVA; means with the same letter are not significantly different ($p > 0.05$). ($F_{3,17} = 36.49$, $p = 0.0001$).

Figure 3. Proteases destroyed the active compound in SGE. Each animal was treated as in figure 1 with 0.5 SGE that had been incubated with buffer alone ($n = 3$), with proteinase K ($n = 2$) or with trypsin ($n = 3$). Buffer without SGE ($n = 3$) was also incubated with either protease (buffer plus enzyme) as a control. The results were analysed by ANOVA; means with the same letter are not significantly different ($p > 0.05$) ($F_{3,7} = 22.72$, $p = 0.0006$).

We used this bioassay to test purified *C. pagurus* MO-IH peptides for bioactivity *in vivo*. As shown in figure 2, SGE (0.5 SG) caused a significant decrease in haemolymph levels of MF ($p < 0.001$). However, purified MO-IH-1 or MO-IH-2 (at 20 pmol per animal, approximately equivalent to the amount of peptide in 1.3 and 2.6 SG, respectively; Wainwright *et al.* (1996)) had no detectable effect on haemolymph levels of MF ($p > 0.05$). This lack of effect was also observed when ESA crabs were treated with a combination of both peptides (not shown).

Figure 4. HPLC chromatogram of SG peptides. The extract of ca. 40 SG was separated by reversed-phase chromatography (see § 2 for details). AUFS, absorbance. Previously characterized peptides (CPRP: CHH precursor-related peptide; MO-IH: mandibular organ inhibiting hormone; CHH: crustacean hyperglycaemic hormone; MIH: moult inhibiting hormone) and the region of MO-IH activity detected in the *in vivo* bioassay are indicated.

The purified MO-IH-1 and MO-IH-2 preparations used in this study were also tested *in vitro* in a parallel study (at a final concentration of 10 nM). These preparations were fully active and depressed MF synthesis *in vitro* to $36 \pm 0.7\%$ ($n = 10$) of the contralateral gland incubated with saline alone.

There are several potential explanations for the observation that SGEs decreased haemolymph levels of MF *in vivo*, while MO-IH-1 and -2 had no activity. However, one obvious explanation is that SGEs contain another compound that regulates the mandibular organ. We tested this hypothesis by determining the chemical nature of the active compound in SGEs. Aliquots of SGE were treated with a protease (either trypsin or proteinase K). As shown in figure 3, incubation of SGE with either enzyme destroyed the ability of the extract to lower MF levels. In other experiments, SGE or purified HPLC fractions were dried and resuspended in methanol or acetonitrile. These treatments, as well as boiling (2 min, results not shown) and extraction of SG with 2 M acetic acid (the procedure used to make the extracts), had no effect on the activity.

To further characterize this compound, SGEs were separated by reversed-phase HPLC using conditions previously used to isolate MO-IH-1 and -2 (figure 4). The eluting fractions were then analysed using the *in vivo* bioassay to identify those that decreased haemolymph levels. Preliminary experiments (not shown) demonstrated that all of the bioactivity eluted between fractions 38 and 48. These fractions were then analysed individually. As shown in figure 5, the peak *in vivo* activity eluted in fractions 40–42. These fractions were also tested for the presence of MO-IH-1 and -2 using a radioimmunoassay developed for both MO-IH peptides. Although there was some overlap between fractions with *in vivo* activity and those with RIA activity, the majority (80%) of the RIA activity eluted in fractions 45–47 that had no *in vivo* activity. The elution times of the RIA active material are similar to those reported previously for MO-IH-1 and -2 (Wainwright *et al.* 1996), the only peptides in the *C. pag-*

Figure 5. Peptides with MO-IH activity *in vivo* were separated from MO-IH-1 and -2 by reversed-phase HPLC. Fractions collected from the chromatogram shown in figure 5 were analysed for activity *in vivo* (using an amount of each fraction equivalent to 0.5 SG per animal). MO-IH immunoreactivity was detected by RIA. Results for the *in vivo* assay are expressed as an inhibition index (100% initial MF) for visual clarity (mean $\pm$ s.e.m., $n = 2$). Black areas indicate immunoreactivity, grey bars indicate *in vivo* activity.

urus SG that affect MF synthesis *in vitro*. Thus, the *in vivo* and *in vitro* activities were chromatographically distinct.

Finally, we investigated the *in vivo* effect of SG fractions on mandibular organ function. HPLC fractions corresponding to the active region identified above were obtained from a bulk extract of SG and pooled. These pooled fractions were used to treat ESA animals. Control animals were treated with saline. Haemolymph samples were removed from each animal before treatment and 3 h later, after which the mandibular organs were removed. Similar to the results obtained previously (see above), treatment with the pooled HPLC fractions caused a significant decrease ($p < 0.0002$) in the haemolymph levels of MF while treatment with saline had no effect (figure 6a). One mandibular organ from each animal was tested for farnesoic acid MeT activity, the final enzyme in the biosynthetic pathway of MF (Claerhout *et al.* 1996; Wainwright *et al.* 1998). MeT activity is known to be down-regulated by MO-IH (Wainwright *et al.* 1998). As shown in figure 6b, the MeT activity in mandibular organs from saline-treated animals was robust. By contrast, the MeT activity in animals treated with the pooled HPLC fractions was undetectable and significantly lower ($p < 0.0001$). Hence, it appears that the *in vivo* active material also regulates the activity of MeT. The level of MF synthesis in the contralateral gland was consistent with this observation. As shown in figure 6c, MF synthesis in mandibular organs from saline-treated animals was sixfold greater ($p < 0.0005$) than the synthesis measured in animals treated with the pooled HPLC fractions. The low level of MF synthesis observed *in vitro* by glands treated with SGE *in vivo* presumably reflects the reactivation of the MF synthetic pathway during the *in vitro* incubation.

4. DISCUSSION

The results presented in this paper demonstrate that SGEs from *C. pagurus* contain a compound(s) that causes a rapid and reversible drop in haemolymph levels of MF in

animals treated *in vivo*. Denaturing conditions (2 M acetic acid, organic solvents, boiling) had no effect on the activity of the SGE. However, the activity was lost when extracts were treated with proteases. These characteristics strongly indicate that the active material is a peptide.

The analysis of SGEs by reversed-phase HPLC demonstrated that the *in vivo* active material eluted in fractions prior to those that contained MO-IH-1 and -2, the only SG compounds that inhibit the synthesis of MF by mandibular organs *in vitro* (Wainwright *et al.* 1996). Nonetheless, the effects of the *in vivo* active material on the mandibular organ function appear to be similar to that of MO-IH-1 and -2 *in vitro*. When animals were injected with pooled HPLC fractions containing the *in vivo* activity, their mandibular organs showed reduced levels of MF synthesis and MeT activity. Thus, it appears that the *in vivo* active material belongs functionally to the group of peptides classified as mandibular organ-inhibiting hormones. To reflect its activity, we will refer to this compound(s) in the rest of the paper as MO-IH_{*in vivo*}.

The MO-IH_{*in vivo*} described in this paper is distinct from peptides that have been previously isolated from *C. pagurus* and *L. emarginata*. Its chemical characteristics are different from MO-IH-1 and -2 found in *C. pagurus*, since it elutes from reversed-phase HPLC in different fractions from these two peptides. In addition, the RIA for *C. pagurus* MO-IH-1 and -2 did not react with the MO-IH_{*in vivo*} peptide(s) described in this paper, indicating that it is immunologically distinct from these previously described peptides. However, the most striking difference is the lack of *in vitro* activity of MO-IH_{*in vivo*} on the mandibular organ. Both MO-IH peptides from *C. pagurus* and the MO-IH isolated from *L. emarginata* are active *in vitro*, demonstrating that they can act directly on the mandibular organ. By contrast, the MO-IH identified does not affect mandibular organ function *in vitro*. Thus, MO-IH_{*in vivo*} appears to belong to a new class of MO-IH peptides that regulate the mandibular organ indirectly. Alternatively, MO-IH_{*in vivo*} may be modified after its release from the SG to a form that affects the mandibular organ directly.

MO-IH_{*in vivo*} was a potent regulator of haemolymph levels of MF when injected into crabs. This effect could reflect several possible actions of this compound, including lowering MF synthesis or stimulating the degradation/excretion of MF. We have not tested the effect of this peptide on these latter processes. However, the *in vivo* treatment of animals with SGE had profound effects on both the MeT activity and the MF synthesis of mandibular organs (figure 6b and c, respectively). These results strongly suggest that one mechanism by which this peptide affects haemolymph levels of MF is the indirect regulation of MF production by the mandibular organ.

MO-IH_{*in vivo*} may affect the mandibular organ by regulating the release of an intermediate compound. The nature of this compound has not been addressed in this study, but there are several possibilities, including the biogenic amines. Although serotonin has been reported to inhibit MF synthesis *in vitro* by mandibular organs from *L. emarginata* (Homola & Laufer 1989), no detectable effect was observed on MF levels when *C. pagurus* was treated with serotonin *in vivo* (results not shown). Nevertheless, several biogenic amines (e.g. octopamine, dopamine and

Figure 6. Treatment of animals *in vivo* with pooled HPLC fractions from a bulk extract of SG decreased MF levels in the haemolymph and MeT and MF synthesis in the mandibular organs. Male crabs were treated *in vivo* with 0.5 SG equivalents in saline or saline alone. Haemolymph samples were taken before, and 3 h after, treatment. At 3 h, the mandibular organs were removed and one gland was analysed for MeT activity and the other for MF synthesis. (a) Saline treatment had no effect on haemolymph levels of MF, while SGE caused a significant decrease ($p < 0.00002$). (b) MeT activity in glands from SG-treated animals was undetectable and significantly lower than the activity observed in glands from saline treated animals ($p < 0.00001$). (c) MF synthesis in glands from SG-treated animals was significantly lower than the level observed in glands from saline treated animals ($p < 0.00005$; *t*-test, $n = 5$ for all groups).

serotonin) have been shown to affect the activity of the corpora allata in many insects (Thompson *et al.* 1990; Pastor *et al.* 1991; Rachinsky 1994), though the response (inhibitory, stimulatory) varies with the stage of the animal (Granger *et al.* 1996). In any case, the potential roles of biogenic amines together with other regulatory factors in the control of the mandibular organ have not yet been clarified.

The discovery of multiple SG peptides with MO-IH activity raises the question of the physiological role of each peptide in the regulation of the mandibular organ. When MO-IH-1 and MO-IH-2 (total amount, 20 pmol) were injected into *C. pagurus* *in vivo*, they had no detectable effect on haemolymph MF levels. Since the total MO-IH content of two SGs is 30 pmol (Wainwright *et al.* 1996), it would appear unlikely that sufficient MO-IH-1 or -2 could be released by the SG to affect mandibular organ function. We suspect that higher doses (greater than 50 pmol) of MO-IH-1 and -2 might have an effect *in vivo*. However, such doses were not tested because they seemed pharmacological. In any case, the absence of data on the fate and clearance rates of these peptides makes it impossible to relate the amounts of these peptides in the SG to general haemolymph concentrations. In addition, the haemolymph concentration may not necessarily reflect the localized MO-IH concentration at the surface of the mandibular organs. Finally, it is possible that purified MO-IH may undergo rapid degradation when injected into the animal, whereas the material released *in vivo* may be protected by other substances from the SG or in the haemolymph. Indeed, there is an apparent paucity of reports describing the effects of treating crustaceans *in vivo* with purified neuropeptides (Chang *et al.* 1990; Soye *et al.* 1991; Liu & Laufer 1996; Grève *et al.* 1999).

While the effects of *Libinia* MO-IH on haemolymph levels of MF have not been reported, this MO-IH does

have CHH activity *in vivo*, a finding that is consistent with the similarity of its amino-acid sequence to peptides in the CHH subfamily of peptides (Liu & Laufer 1996). It has previously been shown that CHH from *C. maenas* will inhibit MF synthesis by mandibular organs *in vitro* (Wainwright *et al.* 1998). In preliminary studies, we found that treatment of ESA animals of this species with 1 SG equivalent of purified CHH (the gift of Dr Simon Webster, University of Wales, Bangor) lowered MF levels *in vivo* (D. W. Borst and G. Wainwright, unpublished observations). Thus, CHH does appear to affect the activity of mandibular organs of this species when administered both *in vitro* and *in vivo*. However, interpretation of the results *in vivo* could be complicated since CHH affects glucose metabolism, which could conceivably affect the acetate precursor pools available for MF synthesis (Sutherland & Feyereisen 1996).

Whereas a case can be made that CHHs have a role in regulating the mandibular organs of some crustaceans, this is apparently not a universal role for CHH since it has no detectable effect *in vitro* on MF synthesis by mandibular organs from *C. pagurus* (Wainwright *et al.* 1996, 1998) or haemolymph levels of MF *in vivo* (this study). However, it seems unlikely that CHH has a physiological role in regulating the mandibular organs of *C. maenas* or *L. emarginata*. In those species in which haemolymph levels of CHH have been measured (*C. pagurus*, Webster (1996); and *H. americanus*, Chang *et al.* (1998)), the concentration of this peptide is elevated by stress, rising from less than 10 pM in unstressed animals to *ca.* 50 pM in stressed animals. However, stress not only causes an increase in CHH levels, but it has also been shown to elevate MF levels in both *C. maenas* and *L. emarginata* (Lovett *et al.* 1997; Ogan *et al.* 1997, respectively). Clearly, it is difficult to comprehend how CHH can act as a MO-IH if its levels rise at the same time as MF levels.

It is difficult to compare the relative potencies of the *C. pagurus* MO-IHs and the MO-IH_{in vivo} in inhibition of MF production, since they are not reciprocally active *in vivo* and *in vitro* and the MO-IH_{in vivo} has not been purified. However, the direct action of MO-IH-1 and -2 in *C. pagurus* and the MO-IH in *L. emarginata* on MF synthesis *in vitro* and the changes in tissue sensitivity observed in *C. pagurus* during vitellogenesis suggest that they have some role in the regulation of this tissue. Sorting out the relationships between MO-IH-1 and -2 and the new MO-IH peptide (MO-IH_{in vivo}) described in this paper will provide a more detailed view of how this crustacean endocrine system is regulated.

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