Identification of methyl farnesoate in the cypris larva of the barnacle, *Balanus amphitrite*, and its role as a juvenile hormone

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Abstract

Previous investigations have shown that insect juvenile hormone (JH) and its analogues induce precocious metamorphosis of barnacle cypris larvae. In the present study, methyl farnesoate (MF; structurally identical to JH III, except for the absence of an epoxide group) has been shown to have a concentration-dependent effect on the development of cyprids of the barnacle *Balanus amphitrite*. Analysis of cypris extracts by gas chromatography–mass spectrometry with selected ion monitoring (GC–MS–SIM) confirmed the presence of endogenous MF. These data provide evidence that MF functions as a juvenilizing hormone in barnacle cyprids, an effect that hitherto has not been noted. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Barnacles, like most other invertebrates, proceed through a complex programme of larval development prior to metamorphosis to the juvenile. Larval development involves six planktonic nauplius stages followed by the lecithotrophic cypris stage. The cyprid, which is specialised to locate a suitable site for attachment and metamorphosis, has long been likened to the pupal stage in insects (Darwin, 1854). In barnacles, metamorphosis from the larval to juvenile form occurs at a single moult (Anderson, 1994). However, the pupal stage analogs has not been supported by investigations on the hormonal control of cypris metamorphosis (Clare, 1995). Gomez et al. (1973) and others (reviewed in Crisp, 1984a) have shown that insect juvenile hormone (JH) and its analogues, which prevent metamorphosis in insects (Slama, 1995), induce precocious metamorphosis in barnacles.

Methyl farnesoate (MF), the unepoxidised form of JH III, has attracted considerable attention in recent years as a potential crustacean hormone (Homola and Chang, 1997). MF was first isolated from the spider crab, *Libinia emarginata* (Laufer et al., 1987), and has since been detected in the hemolymph of several crustaceans (Homola and Chang, 1997). Several roles for this hormone have been proposed, including the control of reproduction (Laufer et al., 1992, 1993), but a functional equivalence to insect JH is still open to conjecture (Homola and Chang, 1997). It has been proposed that MF is involved in crustacean larval development (Borst et al., 1987; Ahl and Brown, 1990; Abdu et al., 1998), including that of barnacles (Yamamoto et al., 1997). The latter study presented evidence that exogenous MF induces metamorphosis of barnacle cyprids without prior attachment, in line with prior work on JH and its analogues (reviewed in Clare, 1995). Although a full structural characterisation of endogenous cyprid MF has yet to be achieved (cf. Yamamoto et al., 1997), the foregoing combined evidence on the effects of exogenous JH and MF on barnacle cyprids suggest that MF may not function as a juvenile hormone in this system.

The present investigation was undertaken to re-examine the role of a juvenilizing hormone in barnacle larval development.
development. The main objectives were to determine whether MF had a role in cypris development, through bioassay, and whether MF could be detected in larval extracts.

2. Materials and methods

2.1. Bioassays

Larvae of Balanus amphitrite were reared in the laboratory to the cypris stage (Clare, 1996). Those larvae that were not to be used immediately were stored at 6°C. JH III, all-trans-MF, cis,trans-MF (2,3-cis, 6,7-trans) and 73% all-trans-MF:27% cis,trans-MF stocks were made up in methanol, then diluted to test concentrations in 0.45 μm-filtered sea water. The final concentration of methanol was ≤1 ppt. Assays were carried out in polystyrene (Falcon 1006) Petri dishes, containing 5 ml of test solution. Controls were exposed to 1 ppt methanol in seawater. Ten cyprids were added to each dish, with five replicates per treatment. The dishes were then incubated at 28°C and observed at 24-h intervals over 5 days. Data were converted to percentage metamorphosis, arcsine transformed, and analysed using a one-way analysis of variance, followed by Dunnett’s test (Instat; Sigma Chemical Co.). Each time point was analysed separately. An α value of ≤0.05 was considered significant.

2.1.1. Pulse exposure of cyprids to MF

Assays with 0.1 μM MF were prepared as described above (Section 2.1). Following a 6-h exposure, cyprids were rinsed thoroughly in seawater and were transferred to clean Petri dishes containing fresh seawater.

2.1.2. Bioassay of cypris extracts

Extracts of 2000 cyprids were prepared as described below (Section 2.2). The dried residues were taken up in methanol and diluted to test concentrations (0.73, 7.3 and 73 cyprid equivalents/ml) in seawater. Assays were performed as described above (Section 2.1)

2.2. Extraction of MF

Dry-blotted cyprids were extracted according to a published method (Borst and Tsukimura, 1991). Batches of at least 10 000 whole cyprids were gently homogenised, on ice, in 2ml 0.9% NaCl, with 2 ml acetonitrile and 100 ng cis,trans-MF as an internal standard. The homogenate was then partitioned three times against hexane (0.5 ml). The hexane fractions were combined, dried under oxygen-free nitrogen, and dissolved in hexane before analysis for MF.

2.2.1. Recovery of MF

In initial work, recovery of MF into the hexane layer of the triphasic partition was determined following addition of a known amount of [12-3H]MF at the beginning of extraction.

2.3. Analysis of cypris extracts

The samples were purified by HPLC on a silica column (25 cmx4.6 mm, 5 μm; Rainin) eluted isocratically at a flow rate of 0.7 ml/min with 1% (v/v) diethyl ether in 50% water-saturated hexane with u.v. detection at 214 nm. The samples were then analysed by gas chromatography–mass spectrometry with selected ion monitoring (GC–MS–SIM) using electron impact ionisation on a Hewlett Packard HP5890 Series II gas chromatograph linked to a VG Quattro mass spectrometer. For this, the MF-containing fraction was evaporated to dryness, resuspended in hexane, and as much as possible of the sample (1 μl) was injected on-column (SGE BP20 capillary column, 25 m×0.32 mm i.d; helium carrier gas pressure, 12 psi) at 50°C, and the temperature of the oven was raised to 220°C at 8°C/min. The ions used for selected ion monitoring were the molecular ion m/z 250, as well as fragment ions at m/z 207, 121 and 69, according to Wainwright et al. (1996). Sixteen extracts, of either day 0 or day 3 cyprids, were analysed for the presence of MF. MF was detected in seven of these samples. The limit of detection for the method used is 100 pg MF.

3. Results

3.1. Bioassays

In each of the bioassays described below, no abnormalities or unattached juveniles were observed in the control treatments.

3.1.1. Effect of JH III

Percent metamorphosis of cyprids exposed to JH III, at concentrations of 1–100 μM, was significantly higher (P<0.05) than that of the controls, and many of the resulting juveniles were abnormal (data not shown). The effect of 0.1 μM JH III, however, was equivocal. In three of the five replicate assays an inhibition of metamorphosis was observed, while no effect was observed in the remaining two assays.

3.1.2. Effect of 73% all-trans-MF:27% cis,trans-MF

Percent metamorphosis of day 0, 3 (Fig. 1) and 7 cyprids exposed to concentrations of 1 and 10 μM MF (73% all-trans-MF:27% cis,trans-MF) was significantly (P<0.01) higher than that of the controls. However, none of the resulting juveniles had attached to the substratum prior to metamorphosis. Fifty percent and 30%
of the juveniles that formed in 1 μM and 10 μM MF, respectively, were abnormal. Most of the abnormal individuals were intermediate cyprid–juvenile forms. Percent metamorphosis of day 0, 3 and 7 cyprids exposed to 0.1 μM MF was significantly (P<0.05) lower than that of the controls. Cyprids in which metamorphosis was inhibited continued to swim normally and no abnormalities were observed for the duration of the experiment. The inhibition of metamorphosis became more apparent over time and was most notable in day 3 cyprids, where after 120 h, 60% of the controls had attached and metamorphosed compared to only 10% of cyprids exposed to 0.1 μM MF. Thus, it is clear that the effect of a low concentration of MF (0.1 μM) on cyprid metamorphosis is quite different from that of high concentrations (1 and 10 μM; Fig. 1). Furthermore, the effects of the latter concentrations are clearly more rapid than that of 0.1 μM MF.

3.1.3. Effects of all-trans-MF and 100% cis,trans-MF
Percent metamorphosis, occurrence of abnormalities and failure to undergo attachment in cyprids exposed to 0.1 to 10 μM all-trans-MF (Fig. 2) was consistent with that of cyprids exposed to the same concentrations of mixed isomer MF (73% all-trans-MF:27% cis,trans-MF). Cyprids treated with cis,trans-MF did not exhibit effects significantly different from untreated controls (P>0.05) with any of the concentrations tested over the range of 0.01–10 μM.

3.1.4. Pulse exposure
Cyprids which had been exposed to 0.1 μM MF (73% all-trans-MF:27% cis,trans-MF) for 6 h began normal attachment and metamorphosis within 24 h of their transfer to fresh seawater (Fig. 3).
3.1.5. Bioassay of cypris extracts

A trend of inhibition of metamorphosis with increasing concentrations of cypris extracts was noted but the results were not significant (P>0.05). At the highest concentration tested, 73 cypris equivalents/ml, a small number of individuals underwent metamorphosis without prior attachment to the substratum (data not shown).

3.2. Analysis of cypris extracts

Analysis of cypris extracts by GC–MS–SIM showed chromatograms with peaks corresponding to the molecular ion (m/z 250) and three characteristic MF fragment ions with the same retention time as all-trans-MF (Fig. 4). The calculated MF titres in cypris extracts ranged from 0.002 to 0.1 μM (n=6) with one exception where it was appreciably higher. Preliminary indications show that MF titres were generally lower in day 3 cyprids compared to those of day 0 cyprids. Percent recovery of MF from cyprid samples was consistently 81% (n=3).

4. Discussion

Metamorphosis of day 0, 3, and 7 cyprids was induced significantly by exposure to JH III and 73% all-trans-MF:27% cis,trans-MF (see Fig. 1) at the higher concentrations tested. Moreover, the juveniles which resulted had all failed to attach to the substratum prior to metamorphosis and many were abnormal; most individuals were intermediate cyprid–juvenile forms. These results are in agreement with previous reports of precocious metamorphosis induced by JH and its analogues (reviewed in Crisp, 1984a; Abdu et al., 1998).

The results of exposing cyprids to 0.1 μM JH III were equivocal. In three of the five replicate assays, an inhibition of cypris metamorphosis was observed, while no effect was seen in the other two replicate assays. When cyprids were exposed to 0.1 μM MF (73% all-trans-MF:27% cis,trans-MF), however, an inhibition of metamorphosis was observed. The natural isomer of crustacean MF is all-trans-MF (Laufer et al., 1987). With respect to the concentrations of MF used in these assays, there is always a possibility that some MF (like JH) would stick to the assay container and, hence, the effective maximum net concentrations of MF available to the animals are those added to the medium. When the effects of this isomer were compared to the non-natural isomer, cis,trans-MF, only the former inhibited metamorphosis. To determine whether the inhibitory concentration of MF adversely affected the cyprids, day 3 cyprids were exposed to 0.1 μM MF for a 6-h period before being removed to MF-free seawater for the remainder of the experiment. The results (Fig. 3) clearly demonstrate that continued exposure to 0.1 μM MF prevents settlement and metamorphosis of cyprids and that pulse exposure to this concentration does not adversely affect the cyprids. This observation could be explained on the basis that we have estimated the concentration of MF in day 0 cyprids to be approximately 0.1 μM (see below), but that the titres of MF apparently fall very rapidly during day 0, to undetectable levels. Thus, if we assume that the high titre of MF in day 0 cyprids compared to day 3 cyprids is sufficient to prevent the onset of settlement and metamorphosis, application of a similar concentration of MF to day 3 cyprids may likewise prevent the onset of settlement and metamorphosis. This indeed appears to be the case, namely physiologically-relevant concentrations of MF prevent day 3 cyprids from settling and metamorphosing (Figs. 1–3). This is entirely consistent with MF acting as a “status quo” or juvenile hormone. These data provide the first indication that precocious metamorphosis may be a result of assaying non-physiological rather than physiological concentrations of
MF and JH and that at least one potential function of MF in cyprids may be to control the nature of the metamorphic moult, a role parallel to that of JH in insects.

The inhibitory and stimulatory effects of cypris extracts in bioassay prompted analysis of extracts by GC–MS–SIM. Selected ion chromatograms were obtained with peaks corresponding to the molecular ion \((m/z\ 250)\) and three characteristic MF fragment ions \((\text{Wainwright et al., 1996})\) with the same retention time as all-\(trans\)-MF, the natural isomer in other crustacean species. This provides strong evidence for the presence of endogenous MF in cypris extracts, in support of earlier findings by \(\text{Yamamoto et al., (1997)}\). Furthermore, the estimated cypris MF titres were physiologically realistic in relation to the bioassay results. The data also suggested a decrease in MF titre with cypris age, but a more detailed analysis will be required to confirm this trend.

It is unclear why high concentrations of MF should induce precocious metamorphosis if MF is a juvenilizing hormone. One possibility that merits investigation is the interaction of MF with the moult hormone 20-hydroxyecdysone \((20E)\). \(\text{Cheung, (1974)}\) reported that an abnormal metamorphosis of \(\text{Balanus eburneus}\) cyprids was promoted by exposure to \(20E\). Since overproduction of ecdysteroid \((\text{hyperecdysonism})\) has previously been corroborated in the crabs \(\text{Carcinus maenas}\) and \(\text{Cancer pagurus}\) \((\text{P.G. Withers, G. Wainwright, H.H. Rees and S.G. Webster, unpublished results})\). Furthermore, JH has been reported to stimulate \(20E\) production in some insects \((\text{e.g. in } \text{Trichoplusia ni}; \text{Jones et al., 1986})\). Although no information is available in barnacles regarding any potential interaction of MF with \(20E\), one possible explanation of precocious metamorphosis, therefore, is that exposing cyprids to a high concentration of MF may have led to an increase in the \(20E\) titre, which, in turn, initiated metamorphosis. Alternatively, incomplete moult and production of larval intermediate stages in response to high concentrations of MF might also be explained in terms of a reduction in \(20E\) titres, as has been demonstrated following JH application in \(\text{Drosophila melanogaster}\) \((\text{Richard and Gilbert, 1991})\). Clearly, studies on cypris \(20E\) titres and the possible relationship between this ecdysteroid and MF will now be required. One further possibility is that these abnormalities may be attributed to precocious muscle degeneration, abnormal water absorption following the moult and/or toxic effects.

Although previous studies have demonstrated an MF-induced delay \((\text{Borst et al., 1987}; \text{Ahl and Brown, 1990})\) or disruption \((\text{Abdu et al., 1998})\) in larval crustacean development, the present study provides the first evidence of a juvenilizing effect of MF on metamorphosis from the larva to the juvenile stage in crustaceans. We conclude that MF is a juvenile hormone in barnacles. The current demonstration of the occurrence of MF is in line with the suggestion that adult barnacles may be capable of MF synthesis \((\text{Lauffer and Landau, 1991})\).

Another role attributed to JH and MF in insects and crustaceans, respectively, is in reproduction \((\text{Homola and Chang, 1997})\). Taken together with what now appears to be a comparable function in delaying metamorphosis, JH and MF may have similar functions in arthropods. In particular, our results suggest that the regulation of development of larval barnacles is akin to that of holometabolous insects where there is also a relatively dramatic metamorphosis. In this respect, Darwin, whose monumental contribution to barnacle biology was not without error \((\text{Crisp, 1984b})\), may have been more characteristically astute when he likened the barnacle cyprid to an insect pupa.

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