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Hepoxilins and trioxilins in barnacles: an analysis of their potential roles in egg hatching and larval settlement

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Summary

The barnacle life cycle has two key stages at which eicosanoids are believed to be involved in cellular communication pathways, namely the hatching of nauplii and the settlement of cypris larvae. Barnacle egg-hatching activity has previously been reported to reside in a variety of eicosanoids, including 8-hydroxyeicosapentaenoic acid and a number of tri-hydroxylated polyunsaturated fatty acid derivatives, the trioxilins. The production of the eicosapentaenoic acid metabolite trioxilin A_4 (8,11,12-trihydroxy-5,9,14,17-eicosatetraenoic acid) by the barnacles *Balanus amphitrite* and *Elminius modestus* was confirmed using a combination of high-performance liquid chromatography and gas chromatography, both linked to mass spectrometry. In addition, both species also generated trioxilin A_3 (8,11,12-trihydroxy-5,9,14-eicosatrienoic acid; an arachidonic acid-derived product), 8,11,12-trihydroxy-9,14,17-eicosatrienoic acid (a ϖ3 analogue of trioxilin A_3; derived from ϖ3 arachidonic acid) and 10,13,14-trihydroxy-4,7,11,16,19-docosapentaenoic acid (a docosahexaenoic acid-derived product). In contrast to earlier reports, trioxilin A_3 had no *E. modestus* egg-hatching activity at any of the concentrations tested (10^{-9}–10^{-6} mol l^{-1}). The unstable epoxide precursor hepoxilin A_3, however, caused significant levels of hatching at 10^{-6} mol l^{-1}. Furthermore, the stable hepoxilin B_3 analogue PBT-3 stimulated hatching at 10^{-7} mol l^{-1}. Neither trioxilin A_3, hepoxilin A_3 or PBT-3 at 0.25–30 μmol l^{-1} served as settlement cues for *B. amphitrite* cypris larvae.

Key words: *Elminius modestus*, *Balanus amphitrite*, barnacle egg-hatching activity, larval settlement, hepoxilin, trioxilin, hepoxilin B_3 stable analogue, PBT-3.

Introduction

Barnacles form a major constituent of the hard fouling organisms that encase man-made structures submerged in the marine environment (Christie and Dalley, 1987). Understanding the factors that regulate their life cycle is crucial towards the development of non-toxic or environmentally benign antifoulants to replace toxic tributyl tin-containing compounds. Following fertilization, barnacle eggs (embryos) are brooded in the mantle cavity of the sessile adults. Upon hatching, the larvae are liberated into the open water and proceed through six planktonic instars and one cyprid instar. During the cyprid or settlement stage, the larvae are believed to use their antennules to detect a suitable substratum upon which to attach and metamorphose into the sessile juvenile barnacle (Crisp, 1976).

The polyunsaturated fatty acid arachidonic acid [AA; 20:4(n-6)] is the precursor of two major families of bioactive lipids, the prostaglandins (PGs), arising through the action of cyclooxygenase(s) (COX), and a series of lipoxygenase (LOX) products that include the leukotrienes and (poly)hydroxylated eicosanoids (Stanley, 2000). Whereas AA is found mainly in mammalian phospholipids (Kühn and Borngraber, 1998), the equivalent predominant C20 polyunsaturated fatty acid present in aquatic invertebrates is usually eicosapentaenoic acid [EPA; 20:5(n-3); Ackman, 1980]. The elongated polyunsaturated fatty acid docosahexaenoic acid [DHA; 22:6(n-3)] is also present in many aquatic animals and acts as a substrate for LOX (Holland et al., 1999) and aspirin-acetylated COX in terrestrial mammals (Serhan et al., 2002).

Within the barnacle life cycle there are two key stages where C20 lipids (eicosanoids) and C22 fatty acid derivatives are thought to be involved in signalling pathways that may influence barnacle development (see review by Clare, 1995).

Firstly, the LOX products 8-hydroxy-5,9,11,14,17-eicosapentaenoic acid (Hill and Holland, 1992; Hill et al., 1988) and a range of tri-hydroxy compounds (Hill et al., 1993; Song et al., 1990a) have been implicated as barnacle egg-hatching factors in both *Elminius modestus* and *Semibalanus balanoides*. *In vivo* studies, using extracted seawater in which barnacles (*S. balanoides* and *Chirona hameri*) had previously liberated their larvae, found trace levels of tri-hydroxy fatty acids, suggesting that these, rather than the mono-hydroxy fatty acid compounds, were the biologically active compounds (Song et al., 1990b). Secondly, Knight et al. (2000) demonstrated that the COX products, prostaglandins E_2 and E_3, caused a dose-dependent inhibition of larval settlement in *Balanus amphitrite*, while indomethacin, a selective COX inhibitor, stimulated the settlement process.

Hepoxilins are epoxide metabolites of AA, EPA and DHA derived through the LOX pathway (Pace-Asciak, 1986; Pace-Asciak et al., 1983; Reynaud and Pace-Asciak, 1997; Fig. 1). In mammals, hepoxilins act primarily on calcium and potassium channels in membranes, which ultimately results in a wide range of functions such as insulin secretion, vascular permeability and vasoconstriction within the aorta and the activation of neutrophils and platelets (for reviews, see Pace-Asciak, 1993, 1994; Pace-Asciak et al., 1999). In invertebrates, their only reported function is in membrane depolarisation, *via* a potassium channel-mediated effect in the neurons of the marine mollusc *Aplysia californica* (Piomelli et al., 1989). Trioxilins are the biologically inert hydrolysis products of the hepoxilins (Fig. 1; Pace-Asciak et al., 1987; Pace-Asciak and Lee, 1989). Hill et al. (1993) reported a number of trioxilins with an 8,11,12-trihydroxy configuration (A-series) produced by *S. balanoides* that commonly appeared in fractions with egg-hatching activity.

The current study investigates the generation of trioxilins by *E. modestus* and *B. amphitrite* using a combination of high-performance liquid chromatography (HPLC) and gas chromatography (GC), both linked to mass spectrometry (MS). The potential functions of their precursors, hepoxilins, as signalling molecules within the egg-hatching and settlement processes of barnacles are also described.

Materials and methods

Animals

Mussels covered by the test barnacle *Elminius modestus* Darwin were removed from the shores around Mumbles Pier, Swansea, UK on spring low tides. Upon return to the laboratory, the animals were maintained at approximately 15°C and 35‰ salinity in a seawater-circulating aquarium for up to 2 weeks prior to use in barnacle egg-hatching activity assays.

Adult *Balanus amphitrite* Darwin (from Beaufort, NC, USA) were maintained in aerated, 10 μm-filtered seawater at 22°C, which was changed every other day, and were fed daily on newly hatched *Artemia* (Sanders, Great Salt Lake, UT, USA). Following larval release, nauplii were cultured in batches (~5000; at 1 larva ml^{-1}) in aerated, ~0.7 μm-filtered seawater at 28°C on a 16 h:8 h L:D cycle and were fed daily on *Skeletonema costatum* (Seasalter Shellfish Ltd, Whitstable, UK) in 1 litre at ~2×10^5 cells ml^{-1}. Antibiotics were administered to the cultures as described by Rittschof et al. (1992). Under these conditions, development to the settlement stage cyprid took 4–5 days. Cyprids were obtained by filtration (retained on a 250 μm mesh) and stored in 0.45 μm-filtered seawater at 6°C until use in larval settlement assays.

Chemicals

Calcium ionophore A23187, arachidonic acid (AA),

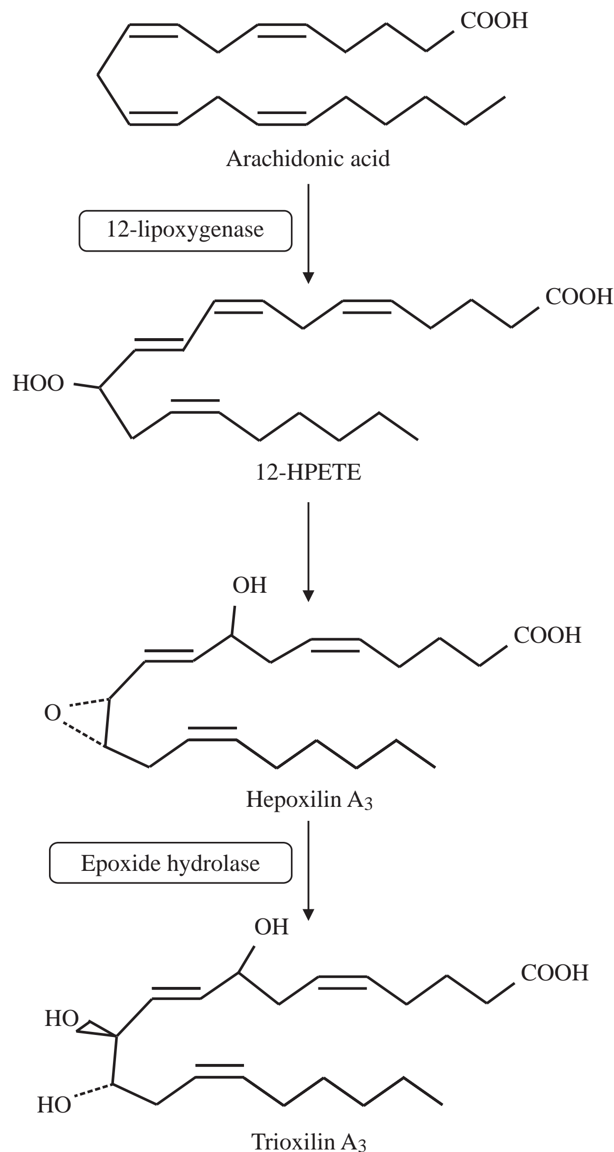

Fig. 1. The biosynthesis of hepoxilin A_3 and its hydrolysis to trioxilin A_3 from arachidonic acid, as reported by Pace-Asciak et al. (1983) in mammalian cells. 12-HPETE, 12-hydroperoxy-5,8,10,13-eicosatetraenoic acid; hepoxilin A_3, 8-hydroxy-11,12-epoxy-5,9,14-eicosatrienoic acid; trioxilin A_3, 8,11,12-trihydroxy-5,9,14-eicosatrienoic acid.

eicosapentaenoic acid (EPA), methoxylamine hydrochloride, bis (trimethylsilyl) trifluoroaceamide and dodecane were purchased from Sigma-Aldrich (Poole, UK). Hepoxilin A_3 was supplied by Biomol Research Laboratories (Plymouth Meeting, PN, USA). The stable hepoxilin B_3 analogue, PBT-3, was prepared as described in Demin and Pace-Asciak (1993). All solvents and acids used in the preparation of material for HPLC-MS and GC-MS were either AnalaR (BDH, Lutterworth, UK) or HPLC grade (Fisher Scientific UK, Loughborough). Ethereal diazomethane was prepared from *N*-methylnitrosotoluene sulphonamide and alcoholic KOH at 65°C using an Aldrich kit (Sigma-Aldrich).

Trioxilin A_3 used in hatching assays and as HPLC-MS and GC-MS standards was prepared by acidification of hepoxilin A_3. Briefly, 100 µl portions of hepoxilin standard (5 µg) were dried under nitrogen, resuspended in 20 µl acetonitrile (20%) and acidified using 50 µl of 0.1% formic acid. Samples were incubated for 20 min, freeze-dried and stored at –80°C in 100 µl methanol until use.

Generation of trioxilins by barnacle tissue

The barnacle extracts used in larval settlement assays and those that were routinely run on HPLC-MS and GC-MS were prepared from known masses (0.7–1.6 g) of soft tissue of either adult barnacles (*E. modestus* or *B. amphitrite*) or known numbers (1000–10 000) of larvae (*B. amphitrite*). Individual species were disrupted on ice in 5 ml of filtered (0.22 µm) seawater. The crude cell preparations were stimulated, for 20 min at 16°C, with 5 μmol l^{-1} calcium ionophore A23187 and either 10 μmol l^{-1} AA, 10 μmol l^{-1} EPA or an equal volume of the fatty acid carrier solvent, ethanol. Debris was removed by centrifugation (2 min, 10 000 ***g***, 4°C) before acidifying the supernatants to pH 3.5 with 10% acetic acid. Samples (4 ml) were loaded onto pre-washed C_{18} Sep-Pak solid phase extraction cartridges (Waters Chromatography, Watford, UK), washed with 5 ml ultra-pure water followed by 1 ml hexane and eluted with methanol (5 ml). Eluants were dried under nitrogen, resuspended in 200 µl methanol and stored at –80°C until preparation for separation by HPLC-MS or GC-MS or use in settlement assays.

Preparation of crude barnacle hatching factor

Crude extracts for use as positive controls in hatching assays were obtained by macerating each adult barnacle species (*E. modestus* or *B. amphitrite*; ~50 g) in 25 ml 0.22 µm-filtered seawater. After 1 h, the liquid fraction was decanted off and the solid fraction homogenised twice (1 h each wash) with 25 ml seawater. Seawater fractions were combined and centrifuged to remove barnacle debris (15 min, 500 ***g***, 4°C). An equal volume of acetone was added to the supernatant, and the precipitated protein was pelleted by centrifugation (10 min, 500 ***g***, 4°C). The supernatant was centrifuged at 14 000 ***g*** for 30 min at 4°C before being filtered (Whatman #1) to remove any remaining particulates. The supernatant was then rotary evaporated (40°C) to remove all acetone, and the aqueous fraction adjusted to contain a final volume of 15% ethanol. The sample was acidified to pH 3.9 with glacial acetic acid, loaded onto a pre-washed (5 ml methanol followed by 5 ml ultra-pure water) C_{18} Sep-Pak, washed with 20 ml ethanol (15%) followed by 20 ml petroleum ether and finally eluted with ethyl acetate. The ethyl acetate eluants were stored at –20°C until use in egg-hatching assays and product identification studies by electron impact gas chromatography–mass spectrometry (GC-EIMS).

High performance liquid chromatography–mass spectrometry (HPLC-MS)

Samples were separated using a Jasco HPLC system on a Spherisorb ODS2 (5 µm, 25 cm×0.46 cm) column (Hichrom) at 1 ml min^{-1} with a 45 min linear gradient of acetonitrile:0.01% formic acid (20:80 to 80:20 v/v). Prior to injection onto the column, samples were dried under N_2 and resuspended in 100 µl of acetonitrile:0.01% formic acid (20:80 v/v) and 5 µl 10% formic acid. The solvent flow passed through a Jasco diode-array detector and was then split; 1/13th of the flow was directed into the electrospray source of a Quattro II triple quadrupole mass spectrometer (Waters Ltd, Elstree, UK) operated in the negative ion mode, and the remainder went to a fraction collector. Fractions (1 min) were collected for 40 min of the HPLC run and, where necessary, were derivatized for GC-EIMS.

Electron impact gas chromatography–mass spectrometry (GC-EIMS)

Samples were converted to their methoxine *O*-trimethylsilyl ether methyl esters for GC-EIMS as described by Knight et al. (1999). Briefly, N_2-dried samples were methoximated with 50 µl of 2% methoxylamine hydrochloride for 30 min at room temperature (RT). After two steps of ethyl acetate extraction, esterification was carried out using ethereal diazomethane (1 h at RT) and the esters treated with 50 µl bis(trimethylsilyl) trifluoroaceamide overnight at RT in an N_2 atmosphere. Samples were resuspended in 20 µl dodecane for GC-EIMS on a Trio 100 instrument (Thermoquest, Hemel Hempstead, UK). Chromatography was carried out on a 30 m DB5 capillary column (Jones Chromatography, Hengoed, UK) using helium as the carrier gas and a temperature gradient of 10°C min^{-1} over 150–320°C. The GC column was routed into the mass spectrometer for analysis by EIMS.

Larval settlement assay

The promotion or inhibition of *B. amphitrite* larval settlement by ionophore-challenged *B. amphitrite* tissue, hepoxilin A_3 and PBT-3 was investigated using freshly moulted cyprids (day 0) and 3-day-old cyprids, respectively. All larval settlement assays were performed using flat-bottomed 24-well culture plates. Each well contained 2 ml of 0.45 µm-filtered seawater, 10 cyprids (day 0 or day 3), and 10 µl of hepoxilin or PBT-3 dissolved in 20% methanol so as to give a final concentration of 0.25, 0.5, 5 or 30 μmol l^{-1}. Control plates were run in parallel, each containing either 10 µl methanol (20%) or 10 µl seawater without hepoxilin A_3 or

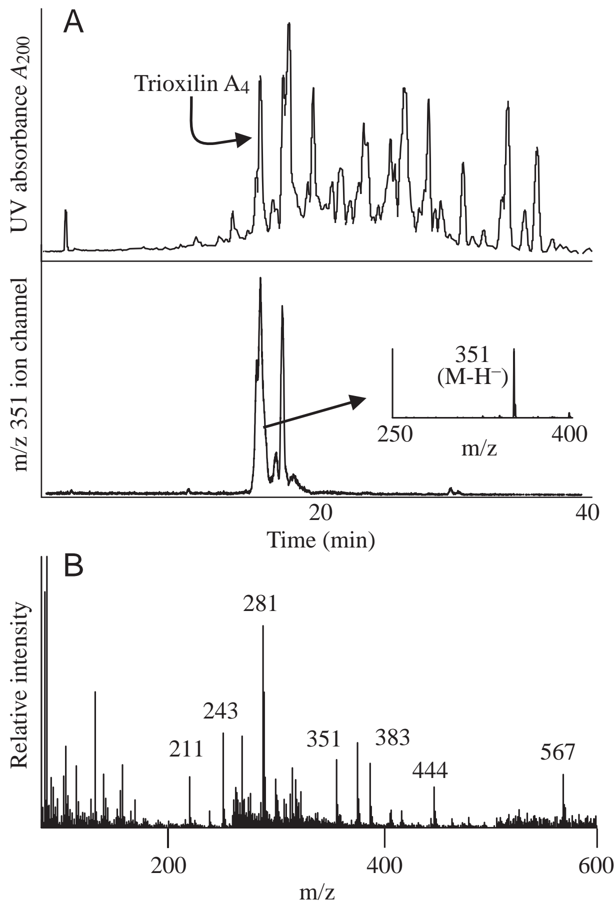

Fig. 2. Ionophore challenge of *E. modestus* generates a number of A_{200} UV-absorbing species, as shown by the HPLC UV profile (A, top panel). Online analysis by negative ion electrospray mass spectrometry (A, bottom panel) highlights the presence of a species eluting at 16 min, which generates an intense M-H⁻ ion at m/z 351 (inset). (B) The electron impact mass spectrum of the *O*-trimethylsilyl ether methyl ester derivative confirms the identity as trioxilin A_4.

PBT-3. All plates were incubated at 28°C in the dark for up to 48 h and were scored for the percentage attachment/ metamorphosis.

Barnacle egg-hatching activity assay

Immediately prior to their use, *E. modestus* were carefully removed from the host mussel shells using a sharp seeker. For each assay, fully developed paired egg masses were removed intact from a single adult barnacle and washed for 1 h in running seawater. One of the egg masses was transferred into 250 µl of filtered (0.45 µm) seawater containing a known concentration of each test compound in an ethanol vehicle. The other egg mass was placed in 250 µl of a control solution containing an equal amount of ethanol (<5%) in seawater. All egg masses were incubated for 15 min at 12°C in separate wells of 48-well culture plates and then fixed by the addition of ~100 µl of 10% seawater formalin. Numbers of hatched (stage 1 nauplii) and unhatched larvae were counted using a

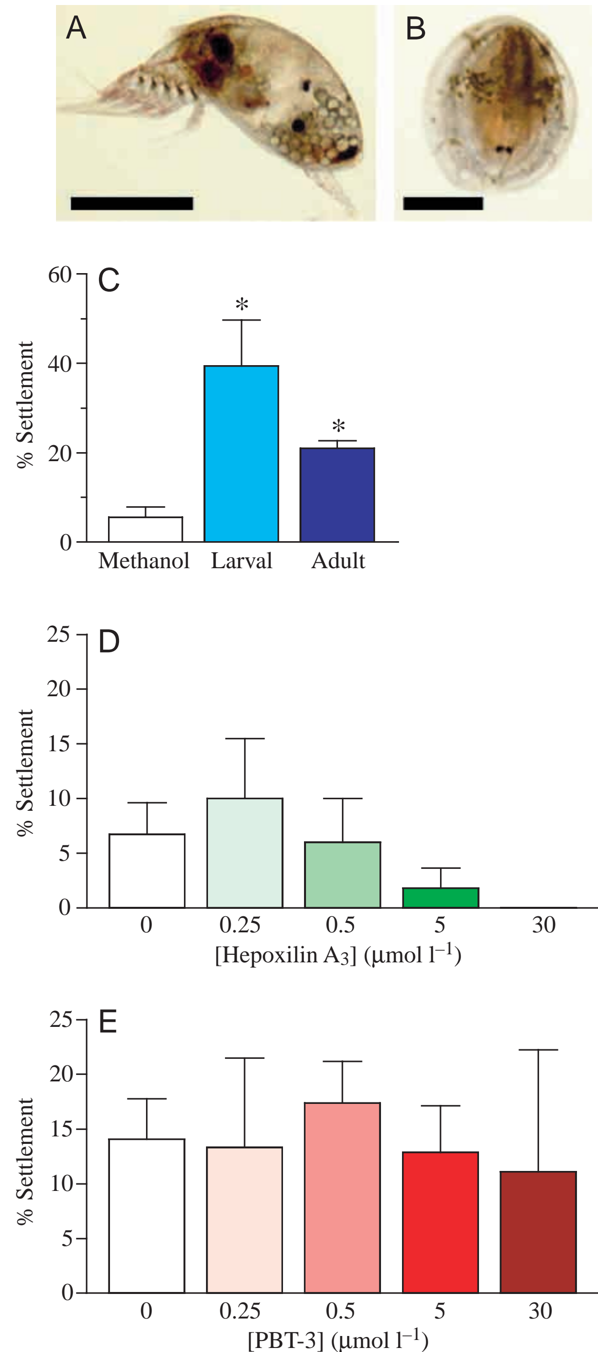

Fig. 3. (A) Unsettled cypris larvae and (B) a recently settled juvenile, as observed in the settlement assays. Scale bars, 0.5 mm. (C) Percentage of settled *B. amphitrite* larvae when exposed to *B. amphitrite* larval extract (equivalent to products from 1000 cyprids well^{-1}) and *B. amphitrite* adult extract (equivalent to products from 0.5 adults well^{-1}). Effect of varying concentrations of (D) hepoxilin A_3 and (E) PBT-3 on the percentage of larval settlement. Values represent arithmetic means + 1 S.E.M., for 10 day-0 cyprids after 48 h (N=3–19). Significant differences $^*P$<0.05 from the methanol controls (open bars).

Sedgewick–Rafter counting chamber. The number of hatched larvae was expressed as a percentage of the total number of

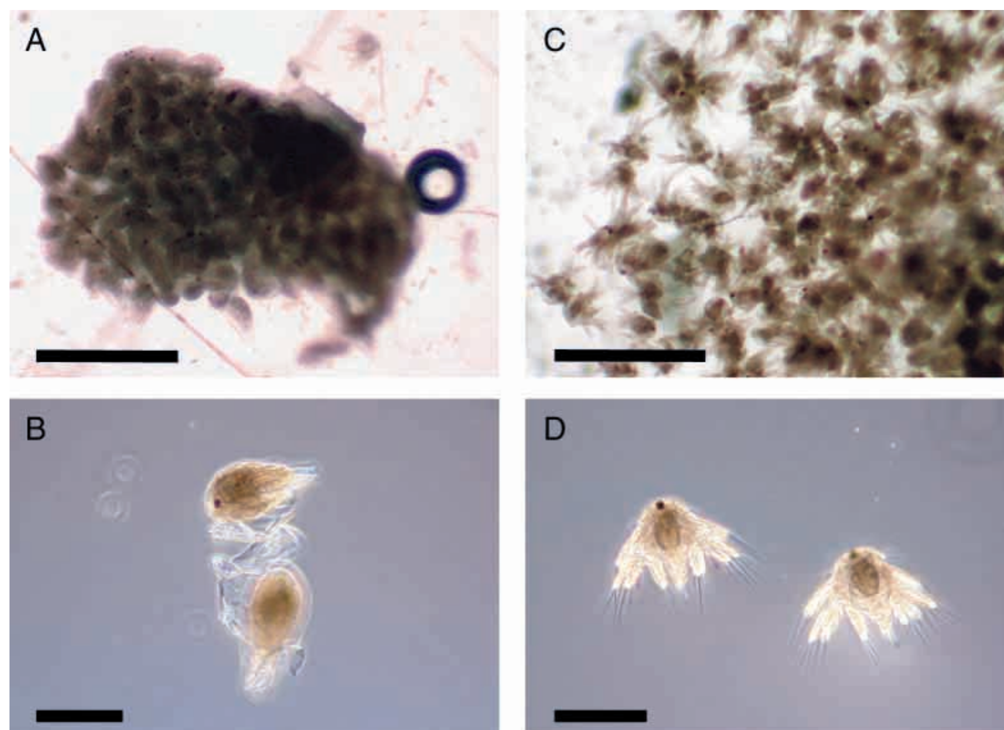

Fig. 4. (A,B) *Elminius modestus* larval egg mass pre-hatching and (C,D) ~5 min after the addition of crude hatching factor. Note the change in the appearance of individual nauplii larvae pre- (B) and post- (D) hatching. Scale bars are 1 mm and 200 μm for A,B and C,D, respectively.

larvae. Any undeveloped larvae found in the centre of the egg mass were excluded from the count. Any unhatched egg masses were tested for hatching viability by addition of the crude hatching factor.

Results

Fatty acids and trioxilin generation in barnacle tissues

Ionophore challenge of both adult *B. amphitrite* and *E. modestus* soft tissues resulted in the formation of a number of A_{200} UV-absorbing species (Fig. 2A). Online analysis by negative ion electrospray MS revealed the presence of EPA, AA and DHA eluting at 34.08 min ($M-H^-$ at m/z 301), 37.66 min ($M-H^-$ at m/z 303) and 36.15 min ($M-H^-$ at m/z 327), respectively, together with some LOX products: mono-hydroxyeicosatetraenoic acids (28.5 min; λ_{max} 234 nm; $M-H^-$ at m/z 319), mono-hydroxyeicosapentaenoic acids (26.2 min; λ_{max} 234 nm; $M-H^-$ at m/z 317) and mono-hydroxydocosahexaenoic acids (28.0 min; λ_{max} 234 nm; $M-H^-$ at m/z 343). Another component present in the mixture that eluted from HPLC at 16 min generated an intense $M-H^-$ ion at m/z 351, corresponding to a molecular mass of 352. The methoxime *O*-trimethylsilylether methyl ester derivative of this compound eluted from the GC at 15.05 min and gave an EI mass spectrum consistent with the EPA-derived trihydroxy fatty acid trioxilin A_4 (8,11,12-trihydroxy-5,9,14,17-eicosatetraenoic acid), with ions at m/z 567 $(M-CH_3)^+$, 477 $(567\text{-TMSOH})^+$, 444 (characteristic rearrangement and loss of ϖ chain), 441 $[M-CH_2.CH{=}CH.(CH_2)_3.COOCH_3]^+$, 383 $(M-CH_3CH_2CH{=}CH.CH_2.CH{=}CH.CH_2\text{-TMSOH})^+$, 371, 282, 281, 243 $[TMSO{=}CH.CH_2.CH{=}CH.(CH_2)_3.COOCH_3]^+$ and 211 $[TMSO{=}CH.CH{=}CH.(CH_2).CH{=}CH.CH_2.CH_3]^+$ (Fig. 2B). The AA-derived product trioxilin A_3 (8,11,12-trihydroxy-5,9,14-eicosatrienoic acid) was also present in the preparation of *E. modestus* hatching factor. It was identified by co-elution with authentic trioxilin A_3 on HPLC-MS, eluting at 18.0 min with an $M-H^-$ ion at m/z 353, and by GC-EIMS, eluting at 14.4 min with ions at m/z 569 $(M-CH_3)^+$, 479 $(569\text{-TMSOH})^+$, 444, 441 $[M-CH_2.CH{=}CH.(CH_2)_3.COOCH_3]^+$, 383 $(M-CH_3CH_2CH{=}CH.CH_2.CH_2CH.CH_2\text{-TMSOH})^+$, 371, 282, 281, 243 $[TMSO{=}CH.CH_2.CH{=}CH.(CH_2)_3.COOCH_3]^+$ and 213 $[TMSO{=}CH.CH{=}CH.(CH_2).CH_2\ CH_2\ CH_2.CH_3]^+$. Incubation of *E. modestus* crude cell extract with either exogenous EPA or AA prior to ionophore challenge caused a marked increase in the production of trioxilin A_4 and trioxilin A_3, respectively. There was no evidence of trioxilin B_3/B_4 production in either barnacle species (based on the reported mass spectra); however, small amounts of an isomeric ϖ-3 trioxilin A_3, 8,11,12-trihydroxy-9,14,17-eicosatrienoic acid, were observed in the GC-MS profile (14.6 min) and identified as arising from ϖ-3 AA by characteristic ions at m/z 569, 479, 463, 446, 422, 385, 371, 351, 282, 281, 243, 211, 147, 121 and 73. The DHA-derived product 10,13,14-trihydroxy-4,7,11,16,19-docosapentaenoic acid was also present, eluting from GC at 17.3 min and generating a spectrum with ions at m/z 608 (M^+), 593, 470, 439, 409, 351, 230, 211, 121 and 73.

Larval settlement

Immediately prior to settlement, the free-swimming cypris larvae of *B. amphitrite* were observed searching the plate surface with their antennules (Fig. 3A). During the settlement process, larvae initially attached to the substratum by their

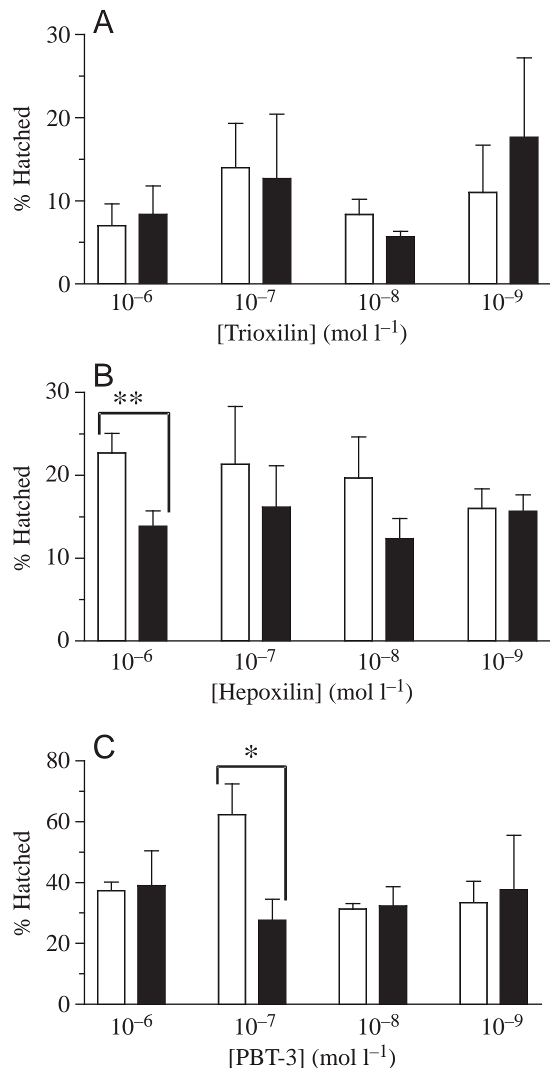

Fig. 5. Percentage of hatched larvae from *Elminius modestus* egg masses when exposed to varying concentrations of (A) trioxilin A_3 (B) hepoxilin A_3 and (C) PBT-3. Open bars indicate each test compound and filled bars indicate the levels of hatching when the partner egg mass was exposed to the ethanol control. Values represent arithmetic means + 1 S.E.M. (N=3–6 for each of the concentrations tested). Asterisks indicate significant differences from ethanol controls at $^*P<0.05$ and $^{**}P<0.01$.

antennules, then head-planted down onto the surface and eventually metamorphosed (moulted) into the new spat (Fig. 3B).

For all experiments, the use of methanol as the solvent carrier (at 0.1% final incubation concentration) had no significant effect on larval settlement of *B. amphitrite* when compared to seawater controls (unpaired *t*-test, $P>0.05$; data not shown). Crude larval (equivalent to products from 1000 cyprids well^{-1}) and adult (equivalent to products from 0.5 adults well^{-1}) extracts from *B. amphitrite* both had a stimulatory effect on larval settlement (unpaired Student's *t*-tests, $P=0.004$ and $P=0.01$ for larvae and adults, respectively; Fig. 3C). Extracts equivalent to the products from ≤200 larvae well^{-1} and ≤0.1 adults well^{-1} had no effect on the settlement of *B. amphitrite* larvae (unpaired Student's *t*-tests, $P>0.05$). Neither hepoxilin A_3 nor a stable analogue of hepoxilin B_3, PBT-3, had a stimulatory (Fig. 3D and 3E, respectively) or inhibitory (data not shown) effect on the settlement of *B. amphitrite* larvae.

Egg-hatching activity

Post-removal from adult *E. modestus*, the pair of egg masses each appeared as a tight aggregation of over 1000 torpedo-shaped embryos embedded in a membrane-bound structure (Fig. 4A,B). Upon addition of the crude egg-hatching factor, the nauplii larvae rapidly emerged (<5 min) from the egg mass with limbs outstretched and actively swimming (Fig. 4C,D). Crude hatching extract derived from *E. modestus* caused 98.17±0.44% (mean ± 1 S.E.M., N=3) of larvae in the egg mass to hatch. Similar levels of hatching (>95%) were observed when *E. modestus* egg masses were exposed to *B. amphitrite* crude hatching extract (data not shown). Authentic trioxilin A_3 had no effect on hatching at any of the concentrations tested (Fig. 5A), while hepoxilin A_3 caused significant numbers of larvae to hatch at the 10^{-6} mol l^{-1} concentration only (paired *t*-test, $P=0.0037$; Fig. 5B). The stable hepoxilin B_3 analogue PBT-3 at a concentration of 10^{-7} mol l^{-1} also had a stimulatory effect on hatching (paired *t*-test, $P=0.0121$; Fig. 5C). A concentration of 10^{-6} mol l^{-1} PBT-3 had no significant effect on larval hatching, although the larvae released were observed to exhibit an inhibition in their swimming behaviour that was not seen with the other concentrations of PBT-3.

Discussion

EPA is the major C20 polyunsaturated fatty acid in the barnacles *B. amphitrite* and *E. modestus* and is metabolised by 8- and 12-LOX activities to form 12-hydroxyeicosapentaenoic and 8-hydroxyeicosapentaenoic acids (Knight et al., 2000). As was seen in *S. balanoides* by Hill et al. (1993), the current study found that, following acidification, *B. amphitrite* tissue extracts contained trioxilin A_3, trioxilin A_4 and 10,13,14-trihydroxy-4,7,11,16,19-docosapentaenoic acid. However, unlike Hill et al. (1993), no evidence was found to support the generation of trioxilin B_4 (10,11,12-trihydroxy-5,8,14,17-eicosatetraenoic acid). The production in *B. amphitrite* of trioxilins A_3 and A_4, together with minor components of ϖ-3 trioxilin A_3 and 10,13,14-trihydroxy-4,7,11,16,19-docosapentaenoic acid, demonstrates that the 12-LOX is active in barnacles across a range of polyunsaturated fatty acids including AA, EPA, DHA and ϖ3 AA. The absence of the epoxide precursors (hepoxilins) is not unexpected as hydrolysis (both enzymatic and non-enzymatic), particularly under the acidic conditions of HPLC, occurs rapidly (Demin et al., 1995).

It has previously been suggested that the trioxilins exhibit biological activity as egg-hatching factors (Hill et al., 1993; Song et al., 1990a). The commercial availability of hepoxilin A_3 allowed us to investigate the effects of hepoxilin A_3 and trioxilin A_3 on egg hatching. In contrast with earlier reports, trioxilin A_3

exhibited no effects in either assay at concentrations up to 10^{-6} mol l^{-1}. The epoxide precursor, hepoxilin A_3, was active at 10^{-6} mol l^{-1}. Hepoxilin A_3 and the stable analogue for hepoxilin B_3, PBT-3, both stimulated the hatching of nauplius larvae at 10^{-6} mol l^{-1} and 10^{-7} mol l^{-1}, respectively. When standardised for the levels of hatching in the ethanol controls, the amount of hatching in the 10^{-7} mol l^{-1} PBT-3 treatment (34.67±3.84%; mean ± 1 S.E.M., *N*=3) was approximately four times greater than that observed for the 10^{-6} mol l^{-1} hepoxilin A_3 treatment (8.83±1.72%, *N*=6). This difference was presumably a reflection of the rapid enzymatic breakdown of hepoxilin A_3 to the inert trioxilin A_3. Despite the evidence for the involvement of hepoxilin in the egg-hatching process, the levels of hatching for the 10^{-7} mol l^{-1} PBT-3 treatment (62.33±10.11%, *N*=3) were much lower than those observed when the egg mass was exposed to the crude hatching factor (98.17±0.44%, *N*=6). The reason for this discrepancy may be due to a number of possibilities. Firstly, it may be that the process of barnacle egg hatching is triggered by a number of factors, such as 8-hydroxyeicosapentaenoic acid (Hill and Holland, 1992; Hill et al., 1988), that may act synergistically to cause larval release. Secondly, given that the major precursor fatty acid in barnacles is EPA, it is possible that the EPA product, hepoxilin A_4, or indeed the DHA-derived 10-hydroxy-13,14-epoxydocosapentaenoic acid, might result in higher levels of larval hatching activity than those observed with the equivalent AA-derived product, hepoxilin A_3. Unfortunately, hepoxilin A_4 (or a stable analogue) is not commercially available, thus the relative effects of the EPA-derived product *versus* the equivalent AA-derived product remain to be determined. Finally, PBT-3 used in the current study is an analogue for hepoxilin B_3, a product that was not observed in *E. modestus*. The use of a stable analogue for hepoxilin A_3 in larval hatching assays might show greater activity than observed in the present study.

Mention should also be made of the concentration of hepoxilins required in the current study to potentiate larval hatching. It is questionable whether a concentration as high as 10^{-6}–10^{-7} mol l^{-1} hepoxilin would exist naturally in the mantle cavity of barnacles where these unhatched larvae normally reside. However, if synergism exists, where a range of bioactive eicosanoids contribute to the natural barnacle hatching-factor activity, then a cumulative effect could lead to concentrations as high as those used in the current study.

No effects on *B. amphitrite* settlement were seen with either hepoxilin A_3 or PBT-3, suggesting that these compounds are not involved in the communication pathways for settlement. However, the involvement of hepoxilins and trioxilins within the barnacle settlement process cannot be completely ruled out. If these compounds function as internal signallers, the concentrations required to permeate the cyprid exoskeleton (cuticle) may exceed those used in the current study (maximal concentration used was 30 μmol l^{-1}). Indeed, Knight et al. (2000) required 50 μmol l^{-1} prostaglandin E_2 or prostaglandin E_3 before significant effects on *B. amphitrite* settlement were observed.

The mechanism of action of hepoxilins and other inducers of larval egg hatching is likely to differ greatly from their potential involvement in the settlement process. Unlike cyprids, which have a thick impervious cuticle, nauplii have only a thin cuticle that has been shown to be highly porous to compounds with molecular masses of <800 Da (Stuart et al., 2002). Thus, in addition to the possibility of interaction with external receptors, compounds such as eicosanoids (~350 Da) may also naturally diffuse through the outer epidermis and make contact with internal receptors. By contrast, the external cues for settlement are generally thought to be peptidic or proteinaceous in character and potentially detected by specific receptors on the cyprid antennules (Clare, 1995). Rather than acting as exogenous signalling compounds, the involvement of eicosanoids in larval settlement is more likely to be within internal signal transduction pathways. An internal role for endogenous eicosanoids is supported by the finding that indomethacin, a selective COX inhibitor, stimulates larval settlement (Knight et al., 2000).

In summary, trioxilins derived from AA, EPA, ϖ3 AA and DHA were produced following ionophore challenge in both *E. modestus* and *B. amphitrite*. Although trioxilin A_3 did not affect egg hatching, the parent epoxide, hepoxilin A_3, was active at 10^{-6} mol l^{-1}. The stable analogue PBT-3 showed activity at 10-fold lower concentrations than hepoxilin A_3, presumably due to its stability to hydrolysis. The egg-hatching activity of barnacle extracts can be ascribed, in part at least, to the hepoxilins. As the extraction protocol involved acidification, which would hydrolyse hepoxilins, it is also likely that some bioactivity may reside with some of the numerous other eicosanoid-like components produced by *E. modestus* and *B. amphitrite*.

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