

# Ecdysteroid concentrations through various life-stages of the meiobenthic harpacticoid copepod, *Amphiascus tenuiremis* and the benthic estuarine amphipod, *Leptocheirus plumulosus*

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## Abstract

Endocrine function in arthropods has principally been characterized in insects and malacostracan crustaceans. However, meiofauna represent the most abundant metazoan marine taxa, with harpacticoid copepods comprising the second most abundant taxon. In addition, their diminutive biomass has made characterization of endocrine components difficult, so little is known about endocrine control of reproduction, molting, and growth in meiofauna. In this study, a sensitive fluorometric enzyme immunoassay (EIA) was utilized to quantify and compare the arthropod molting hormone, 20-hydroxyecdysone (20E), in various life-cycle and developmental stages of a laboratory reared meiobenthic copepod, *Amphiascus tenuiremis*, and in an amphipod, *Leptocheirus plumulosus*. In copepods, gravid females carrying late stage pre-hatch embryos contained significantly more 20E ( $390 \pm 252$  fmol/female) than gravids carrying early (Stage-I) embryos ( $172 \pm 83$  fmol/female). In contrast, ecdysteroid levels in Stage-I *L. plumulosus* gravid females ( $277 \pm 83$  fmol/female) was greater than pre-hatch gravid females ( $146 \pm 42$ ). Stage-I embryos of both copepods ( $19 \pm 10$ ) and amphipods ( $11 \pm 5$  fmol/embryo) possessed lower ecdysteroid content than copepod ( $35 \pm 15$ ) and amphipod ( $43 \pm 33$  fmol/embryo) pre-hatch embryos. Ecdysteroid levels were also assessed in naupliar, juvenile, adult male and non-gravid female copepod life-stages. In addition, ecdysteroids measured in field collected copepod species indicated gravid females possessed ecdysteroid levels similar to gravid *A. tenuiremis*. However, upon normalization of egg sac 20E content by brood size, embryos from larger broods contained lower levels of ecdysteroids when compared to embryos from smaller clutch sizes—indicating an inverse embryo/ecdyteroid relationship may exist across species.

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## 1. Introduction

Considerable research has concentrated on understanding endocrine function and regulation in arthropods, yet most studies have focused on insect species. Due to this disparity, much less is known with regard to endocrine regulation of growth, development, and reproduction in other arthropod classes, such as crustaceans. Comparative observations, however, have suggested both insect and crustacean endocrine systems share a high degree of homology with many similarities in key signaling

molecules and regulatory feed-back mechanisms (Chang, 1993; Horn et al., 1966; King and Siddall, 1969).

One highly conserved endocrine mechanism in arthropods is the hormonal pathway required to stimulate molting. In insects and crustaceans, molting is mediated by ecdysteroids, with predominant forms including the inactive parent compound, ecdysone, and a biologically active metabolite, 20-hydroxyecdysone (20E) (Chang and O'Connor, 1978; Feyereisen et al., 1976). In decapod crustaceans, ecdysone and 20E are synthesized from dietary cholesterol in the Y-organ; a non-neural endocrine gland found in larvae, juveniles, and adults (Chang, 1997; Watson and Spaziani, 1985). Synthesis and secretion are positively regulated by the crustacean juvenile hormone, methyl farnesoate (MF) (Laufer et al.,

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1993), and are under negative control by molt-inhibiting hormone (MIH)—a neuropeptide secreted by the sinus gland/X-organ complex (Mattson and Spaziani, 1985). During macrocrustacean molt cycles, 20E titers typically peak in premolt stages followed by a sharp ecdysteroid decline as ecdysis ensues. Immediately following ecdysis, Y-organ activity declines, and ecdysteroids are maintained at basal levels (Okumura et al., 1992; Skinner, 1985). Ecdysis refers solely to the actual shedding of the old exoskeleton whereas molting refers to the entire process of preparation, shedding, formation, and hardening of the new exoskeleton (Fingerman, 1987).

Ecdysteroids also perform key roles in crustacean reproduction. Removal of the Y-organ has induced arrest of vitellogenesis in amphipods and isopods leading to inhibited vitellin (egg yolk protein) synthesis (Meusy et al., 1977; Souty et al., 1982; Suzuki et al., 1996). Similarly, increased 20E titers have been correlated to increased yolk protein synthesis and uptake in developing oocytes (Blanchet-Tournier, 1982; Gohar and Souty, 1984). Ecdysteroids also can tightly bind to vitellin, suggesting that bound ecdysteroids can be maternally transferred to oocytes (Nirde et al., 1983; Spindler et al., 1980; Subramoniam, 2000; Subramoniam et al., 1999; Wilder et al., 1991). Maternally derived ecdysteroids can then function to stimulate early developmental larval molting until larvae/juveniles generate a Y-organ and synthesize ecdysteroids endogenously (Chang, 1992).

Although crustacean endocrine signaling seems conserved across taxa, interspecies mechanisms for molting and reproduction can be highly variable (for a review, see Charmantier et al., 1997). Furthermore, most of what is currently understood about crustacean endocrine systems has emerged from research on malacostracans (decapods, amphipods, and isopods) (Fingerman, 1987). Yet meiobenthic copepods represent the most abundant crustacean taxon in marine sedimentary systems at  $> 10^{5-6}/\text{m}^2$  sediment (McIntyre, 1969). One explanation for this data gap is that endocrine components have been difficult to characterize with traditional assays due to the diminutive biomass of meiofaunal species (1–10  $\mu\text{g}/\text{individual}$ ). Thus, almost nothing is known about hormonal control of reproduction, molting, growth, metamorphosis and homeostasis in microcrustaceans. Subsequently, in order to provide an introductory characterization of meiobenthic copepod endocrinology, this study used a high sensitivity, fluorescence-based, enzyme immunoassay (EIA) to quantify and compare ecdysteroid titers in various life-stages of the meiobenthic harpacticoid copepod *Amphiascus tenuiremis* and the estuarine malacostracan amphipod, *Leptocheirus plumulosus*. Ecdysteroid content was also measured in field collected copepods in order to evaluate and compare 20E levels across several harpacticoid species.

## 2. Materials and methods

### 2.1. Model crustaceans

#### 2.1.1. *Amphiascus tenuiremis*

Meiobenthic harpacticoid copepods are extremely important in food webs and carbon/nutrient cycling (Hicks and Coull, 1983). Additionally, harpacticoids are reported to comprise 4–95% of the total sediment meiobenthos (Coull et al., 1979; Coull and Wells, 1981) and 11 to 60% of the total phytoplankton meiobenthos (Hicks, 1977); second in abundance only to nematodes in non-deep-sea environments (McIntyre, 1969). *A. tenuiremis* is a diosaccid harpacticoid that normally lives buried in the upper 0.5 cm muddy sediment horizon of intertidal to subtidal estuarine habitats with distribution ranging from the Baltic Sea to the southern Gulf of Mexico (Lang, 1948). It has a generation time of 21 days at 20 °C (Chandler and Green, 1996), and a life-cycle consisting of six naupliar and six copepodite stages with sexual maturity reached after the fifth copepodite stage (Hicks and Coull, 1983). Gravid females extrude dual egg sacs with each sac containing 6–9 embryos in planar arrangement. Additionally, mature *A. tenuiremis* are sexually dimorphic with males having a swollen geniculate segment on their antennae for clasping females during mating and spermatophore transfer. *A. tenuiremis* were cultured for this study in pristine North Inlet, SC, USA muddy sediments on an algal diet (1:1:1 *Dunaliella tertiolecta*:*Isochrysis galbana*:*Phaeodactylum tricorutum*) in a carbon scrubbed recirculating seawater system (after Chandler and Green, 1996) at 30 S, 23 °C, and a 12 h:12 h light:dark cycle.

#### 2.1.2. *Leptocheirus plumulosus*

The benthic estuarine amphipod, *L. plumulosus*, is a burrow-constructing member of the family Aoridae found in subtidal oligohaline and mesohaline estuaries of the United States east coast (Bousfield, 1973; Dewitt et al., 1992). Distribution has been observed from the North Atlantic coast to northern Florida with abundances reaching  $> 2.5 \times 10^4/\text{m}^2$  (Holland et al., 1988). *L. plumulosus* ingests a wide range of estuarine particles by filter feeding on suspended particulate matter and deposit feeding on sediment and detritus (Dewitt et al., 1992). In a laboratory environment, the amphipod has a generation time of 40–45 days and can reach sexual maturity in 3–4 weeks. Once mature, gravid female amphipods can produce at least six marsupial clutches per lifetime, ranging from 5 to 15 neonates/female. Non-gravid amphipods are sexually differentiated by the presence or absence of male penile papillae and female marsupium oostegites (Gray et al., 1998). *L. plumulosus* were cultured for this study in muddy sediments (North Inlet, SC, USA) on a diet of 1:1 *D. tertiolecta*:*I. galbana* at 15 S, 23 °C, and a 12 h:12 h light:dark cycle.

## 2.2. Reagents, immunochemicals, and standards

Anti-20E rabbit polyclonal antisera and 20E-horseradish peroxidase conjugate were obtained from Dr. Tim Kingan (University of California at Riverside, Riverside, CA). Affinity purified  $F_c$ -specific goat anti-rabbit IgG antisera was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). 20E (>99%) used as a standard reference material was purchased from A.G. Scientific (San Diego, CA). All other reagents were purchased from Sigma (St. Louis, MO).

## 2.3. 20-Hydroxyecdysone enzyme immunoassay

Our 20-hydroxyecdysone EIA was modeled after the approach outlined by Kingan (Kingan, 1989; Kingan and Adams, 2000; Kingan et al., 1997), but modified/optimized for microgram of tissue masses via use of a fluorogenic substrate. In brief, 96-well, high-binding black microplates with clear optical bottoms (Corning Costar) were coated 14–16 h at room temperature with 500 ng/well of an  $F_c$ -specific goat anti-rabbit IgG antibody. A concentrated stock of  $F_c$ -specific antisera was diluted in a 10 mM phosphate-buffered saline buffer (PBS; 137 mM NaCl, 2.7 mM KCl, and 10 mM phosphates at pH 7.5) to obtain a final coating volume of 90  $\mu$ l/well. The coating solution was then discarded followed by addition of blocking buffer (25 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, and 0.1% (w/v) bovine serum albumin at pH 7.5; 300  $\mu$ l/well) and incubation for 1 h at room temperature with constant mixing on an orbital shaker (Lab-Line, Melrose Park, IL). The coated and blocked microplates were washed three times with PBS/0.05% Tween 20 (PBST). Fifty microliters of sample or 20E standards (20–4000 pM) were added to wells in duplicate, while 100  $\mu$ l of blocking buffer was used as a blank to account for non-specific binding. Anti-20E rabbit polyclonal antisera was diluted 1:1100 in blocking buffer and added to wells (50  $\mu$ l/well) containing samples and standards followed by addition of 50  $\mu$ l 20E-horseradish peroxidase (20E-HRP) conjugate (1:2200 in blocking buffer) to all wells. The competitive reaction was allowed to equilibrate overnight at 4 °C followed by three washes with PBST. One hundred microliters of QuantaBlu Fluorogenic Peroxidase Substrate (Pierce, Rockford, IL) was supplied to each well and developed for two hours at room temperature while mixing on an orbital shaker. The enzyme/substrate reaction was quenched with addition of 100  $\mu$ l/well QuantaBlu Stop Solution and immediately analyzed using a BIO-TEK FL<sub>x</sub>800 fluorescence microplate reader (BIO-TEK Instruments, Winooski, VT) equipped with KC<sub>Junior</sub> v1.22 software for PC. Fluorescence was detected utilizing an excitation/emission filter set of 340  $\pm$  30 and 420  $\pm$  50 nm, respectively. Additionally, the antisera used here had greatest affinity toward ecdysone and 20E

with minimal cross-reactivity measured for ecdysteroids containing structurally different side chains (ponasterone A) or sterol nuclei (“triol”) (Kingan, 1989). Therefore, all reported ecdysteroid measurements from this study were expressed as 20E equivalents.

## 2.4. 20E EIA substrate analysis

Multiple substrate analysis compared the functionality of colorimetric and fluorescent HRP substrates for EIA detection of 20E. Standard curves were prepared with four replicates per concentration following the above described methodology. For colorimetric detection of 20E, 100  $\mu$ l of 3,3',5,5'-tetramethylbenzidine (TMB) soluble HRP substrate (Pierce, Rockford, IL) was added to each well and developed for 15 min at room temperature while mixing on an orbital shaker. The enzyme/substrate reaction was stopped with addition of 100  $\mu$ l/well 1 N H<sub>2</sub>SO<sub>4</sub> and immediately analyzed using a BIO-TEK Powerwave 200 microplate reader (BIO-TEK Instruments, Winooski, VT) set for maximum absorbance at 450 nm. Fluorometric detection of 20E followed the aforementioned procedure utilizing QuantaBlu Fluorogenic Peroxidase Substrate and Stop Solutions. Substrates were compared by their EC<sub>50</sub>s and linear non-asymptotic slopes, with the EC<sub>50</sub> defined as the concentration, in femtomoles of standard, required for 50% displacement of the 20E-HRP conjugate.

## 2.5. Sample collection from laboratory cultures

Males, gravid females, embryos, nauplii, and copepodites were collected ( $n = 10$  for each life-stage) and sorted by sieve size from laboratory sediment cultures of *A. tenuremis*. Gravid copepods were segregated as to whether brood sacs were early (Stage-I embryos, <24 h old) or late (pre-hatch, ~72 h old) in embryonic development. Brood stages can be easily distinguished by the prevalence of high-density localized yolk proteins in Stage-I brood sacs. Brood sac pairs, each enclosing 6–9 developing embryos, were microdissected from individual females and assayed individually for ecdysteroid concentrations. Adult male and female copepods were collected by sieving across 125 and 90  $\mu$ m steel mesh, while juvenile C-1st and C-4th stage copepodites (C-1s and C-4s) were separated across 90 and 75  $\mu$ m steel mesh. C-4 copepodites were then subdivided into those exhibiting male or female secondary sexual characteristics. Nauplii (<20 h old) were collected and pooled from 120 pre-hatch gravid females incubated in two wells of a 12 well Netwell microplate, equipped with a 74  $\mu$ m polycarbonate mesh filter (Corning Costar) suspended in 0.2  $\mu$ m filtered, 30 S Instant Ocean synthetic seawater.

Mature male, non-gravid female, gravid female, and embryonic *L. plumulosus* ( $n = 10$  for each life-stage) were sorted from laboratory cultures by sieving sedi-

ments and amphipods across a 500  $\mu\text{m}$  steel mesh with 15 S Instant Ocean. Gravid female amphipods were further sorted as to the presence of Stage-I or pre-hatch developmental-stage embryos. Similar to copepods, Stage-I amphipod embryos exhibit densely packed yolk vesicles and less than four cleavage planes. Only gravid females carrying broods of at least five embryos were used for 20E analysis.

### 2.6. Field collection of meiofaunal copepods

Field populations of meiofaunal harpacticoid copepods were sampled at low tide from a pristine salt marsh near Oyster Landing, North Inlet, SC, USA. The top one cm of sediment was carefully collected from a 1 m<sup>2</sup> area of mudflat and immediately passed through a 95  $\mu\text{m}$  sieve. Retained gravid female copepods containing Stage-I embryos were segregated and identified in the laboratory to species. Species identified included *Nannopus palustris* ( $n = 4$ ), *Enhydrosoma propinquum* ( $n = 5$ ), *Stenhelia bifidia* ( $n = 4$ ), and *Microarthridion littorale* ( $n = 6$ ). Gravids of each species (and brood sacs) were then assayed as above for ecdysteroids.

### 2.7. Ecdysteroid extraction

Collected copepods and amphipods were placed in 1.5 ml microcentrifuge tubes and homogenized in 500  $\mu\text{l}$  ice-cold methanol–water (4:1) using a Pellet Pestle tissue grinder (Kontes, Vineland, NJ) followed by vortexing for 30 s. Methanol–water was used for tissue extractions because the solvent mixture has been found to be an effective means of ecdysteroid extraction with an efficiency of greater than 90% (Baldaia et al., 1984; Chang and O'Connor, 1979; Kingan, 1989). Extracted tissues were allowed to incubate 24 h at  $-20^\circ\text{C}$  in order to denature and precipitate out proteins present in the homogenate. Tissues were then centrifuged (Eppendorf) at 12,000 rpm (11752 RCF) for 10 min and the supernatant transferred to a fresh microcentrifuge tube. Two hundred additional microliters of methanol–water was added to the pelleted extracts followed by vortexing and centrifugation, with the resulting supernatant transferred and pooled with its predecessor. All samples were taken to dryness in a vacuum centrifuge (Labconco, Kansas City, MO) and stored at  $-20^\circ\text{C}$  until analysis. Tissue extracts were then dissolved in 200  $\mu\text{l}$  blocking buffer immediately prior to EIA analysis of ecdysteroid concentrations.

### 2.8. Statistical analysis

Statistical analyses of 20E and cholesterol measurements were conducted using Sigma Stat version 2.03 statistical software (Jandel Scientific, San Rafael, CA). Data were first tested for normality ( $p = 0.01$ ); where

failed, logarithmic transformation was performed and analysis repeated. Differences among class means were tested using one-way analysis of variance (ANOVA) with Bonferroni a posteriori multiple comparisons ( $p = 0.05$ ). Standard curve substrate analysis was conducted using a 4-parameter logistic regression modeled in Prism version 2.0 software (GraphPad, San Diego, CA). Differences among substrates were determined by performing a studentized  $t$  test ( $p = 0.05$ ) on the slope and inflection point ( $\text{EC}_{50}$ ) of each curve.

## 3. Results

### 3.1. Standard curve substrate analysis

Multiple substrate analysis resulted in monophasic dose-response curves for both TMB and QuantaBlu substrates (Fig. 1) with respective  $R^2$  goodness of fit indices greater than 0.99. Additionally, the mean QuantaBlu  $\text{EC}_{50}$  of 28.8 ( $\pm 2.2$  SD) femtomoles was significantly lower than the TMB  $\text{EC}_{50}$  of 64.8 ( $\pm 5.6$ ) femtomoles. Similarly, the slope of the QuantaBlu standard curve ( $2.97 \pm 0.24$ ) was significantly decreased from the slope produced by the TMB standard curve ( $3.45 \pm 0.25$ ).

### 3.2. Ecdysteroid quantification

In *A. tenuiremis*, gravid females carrying late stage embryos (with egg sacs removed) possessed 20E levels of  $390 \pm 252$  fmol/copepod (mean  $\pm$  SD), which was significantly greater and more variable than the concentration of  $172 \pm 83$  fmol/copepod measured in gravids carrying early Stage-I embryos (with egg sacs removed) (Fig. 2). Similarly, older, pre-hatch embryos had almost double the 20E content of early Stage-I embryos at  $35 \pm 15$

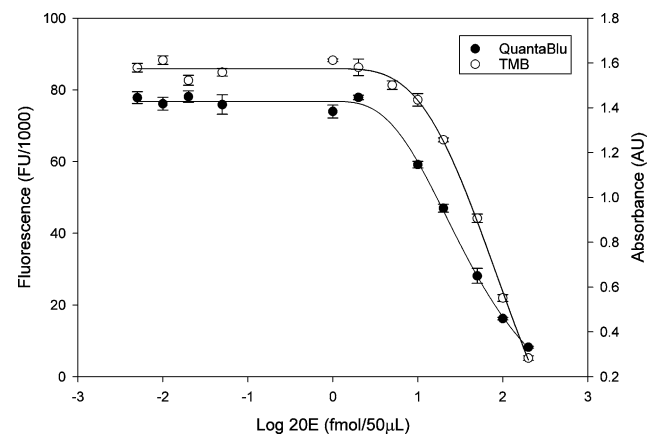


Fig. 1. 20E enzyme immunoassay (EIA) standard curve using TMB ( $\circ$ ) and QuantaBlu ( $\bullet$ ) peroxidase substrates. TMB and QuantaBlu standards are reported as absorbance units (AU) and fluorescent units (FU), respectively. Data are presented as means ( $\pm$ SD).

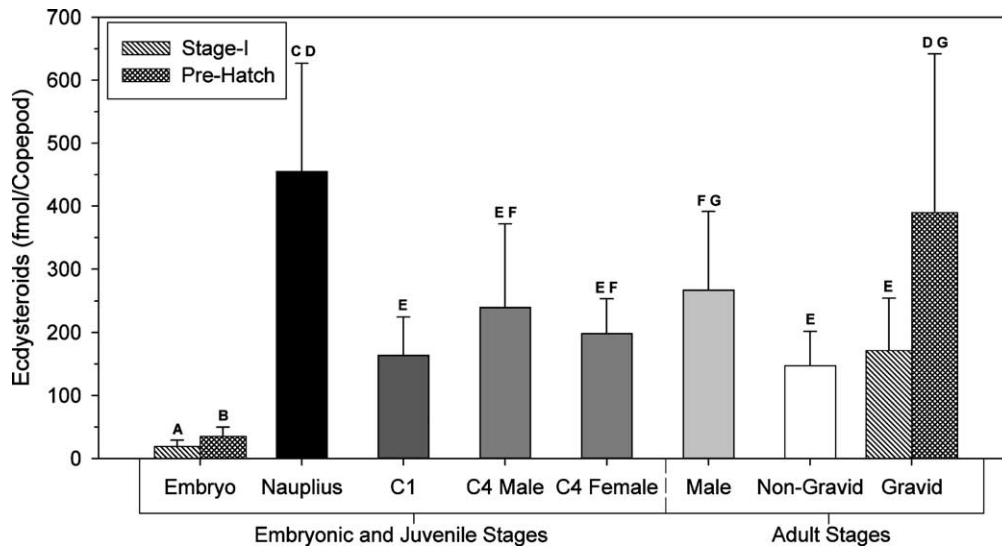


Fig. 2. Ecdysteroid concentrations in male, gravid female, embryo, naupliar, and copepodite *A. tenuiremis*. Embryos and corresponding gravid females were also segregated into early (Stage-I) and late (pre-hatch) developmental stages. Bars with dissimilar letters are significantly different ( $p < 0.05$ ). The power of performed ANOVA was 0.945. Data are presented as means ( $\pm$ SD).

versus  $19 \pm 10$  fmol/embryo, respectively. After hatching, 20E in nauplii (<20 h old) increased sharply to  $448 \pm 189$  fmol/nauplius, while further in development, 20E declined in immature C-1 stage copepodites (~6 days old) to  $164 \pm 60$  fmol/copepodite. In addition, immature male ( $240 \pm 132$  fmol/copepodite) and female ( $199 \pm 55$  fmol/copepodite) C-4 stage copepodites (~15 days old) exhibiting secondary sexual characteristics possessed slightly increased 20E levels compared to those observed in C-1s. No significant differences between copepodite stages were observed, however naupliar 20E was significantly higher than Stage-I gravid females, Stage-I and pre-hatch embryos, and copepodite stages. Ecdysteroids measured in adult male and non-gravid female copepods averaged  $268 \pm 124$  and  $147 \pm 55$  fmol/copepod, respectively, with males having 20E levels significantly greater than all life-stages with the exception of Stage-I gravid females, nauplii, and C-4 copepodites.

Ecdysteroid analysis of gravid and non-gravid female, embryonic, and adult male *L. plumulosus* was evaluated (Fig. 3). In this amphipod, Stage-I gravid females ( $277 \pm 83$  fmol/female) were almost double in 20E content than pre-hatch gravids ( $146 \pm 42$  fmol/female), which is in contrast to the copepod distribution pattern described above. Pre-hatch embryos had significantly greater 20E content than Stage-I embryos at  $43 \pm 33$  versus  $11 \pm 5$  fmol/embryo, respectively. Male amphipods had a 20E level of  $350 \pm 99$  fmol/male, which was numerically superior to all other life-stages. This finding was statistically significant for males compared to both embryonic stages and to pre-hatch gravid females. 20E among individual non-gravid females was highly variable at  $252 \pm 137$  fmol/female, thus a significant differ-

ence was observed only against Stage-I and pre-hatch embryos.

### 3.3. Ecdysteroid concentrations in field collected harpacticoid copepods

In addition to *A. tenuiremis*, ecdysteroid content was also measured in Stage-I gravid females (with egg sacs removed) and Stage-I egg sacs from *N. palustris* ( $n = 4$ ), *E. propinquum* ( $n = 5$ ), *S. bifidia* ( $n = 4$ ), and *M. littorale* ( $n = 6$ ) (Table 1). 20E concentrations in Stage-I females ranged from 121 (*S. bifidia*) to 172 (*A. tenuiremis*) fmol/copepod, however, no significant differences were ob-

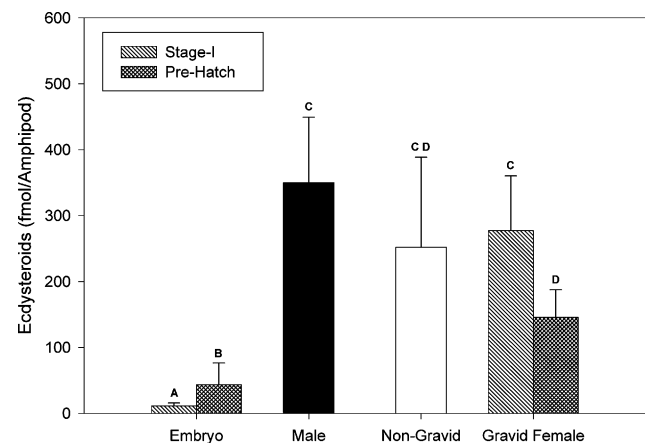


Fig. 3. Ecdysteroid concentrations in *L. plumulosus* adult males, non-gravid females, gravid females, and embryos. Embryos and corresponding gravid females were further segregated into early (Stage-I) and late (pre-hatch) developmental stages. Bars with dissimilar letters are significantly different ( $p < 0.05$ ). The power of performed ANOVA was 0.829. Data are presented as means ( $\pm$ SD).

Table 1  
Ecdysteroid levels in various meiobenthic copepod species<sup>a</sup>

Species	Female (fmol/copepod)	Egg sac (fmol)	Embryos <sup>b</sup> (per egg sac)	Embryo (fmol/embryo)
<i>N. palustris</i> (n = 4)	145 (67)	88 (32)	26.0 (4.1)	3.5 (1.5)
<i>E. propinquum</i> (n = 5)	158 (88)	264 (142)	10.5 (1.0)	21.0 (12.0)*
<i>S. bifidia</i> (n = 4)	121 (93)	137 (63)	5.0 (1.2)	27.6 (10.0)*
<i>M. littorale</i> (n = 6)	153 (54)	159 (23)	12.7 (1.6)	12.7 (2.5)*
<i>A. tenuiremis</i> (n = 10)	172 (83)	173 (89)	9.6 (2.7)	19.1 (10.0)*

<sup>a</sup> All data are reported as means (SD).

<sup>b</sup> Embryos/egg sac significantly different ( $p < 0.05$ ) for all species except between *E. propinquum* and *A. tenuiremis*.

\* Values are significantly different from *N. palustris*.

served. Additionally, whole copepod egg sacs possessed ecdysteroid levels ranging from 88 (*N. palustris*) to 264 (*E. propinquum*) fmol/egg sac, although, no significant differences were seen due to moderate within-species variability. In contrast, differences in brood sizes across copepod species were seen with values ranging from 5 (*S. bifidia*) to 26 (*N. palustris*) embryos/egg sac. With the exception of *E. propinquum* and *A. tenuiremis*, broods from each species all contained significantly different numbers of embryos. Normalization of total brood ecdysteroids to the number of embryos measured per brood resulted in a calculated 20E content ranging from 3.5 (*N. palustris*) to 27.6 (*S. bifidia*) fmol/embryo. 20E in *N. palustris* embryos was significantly lower than other copepod embryos, but no other significant differences in embryonic ecdysteroids were observed.

#### 4. Discussion

The dynamic synthesis, regulation, and metabolism of ecdysteroids required for molting, reproduction, and development can result in widely variable 20E tissue concentrations with regard to species, life-stage, age, and gender. Subsequently, a highly sensitive and precise enzyme immunoassay, capable of detecting femtomolar ecdysteroid concentrations from a single copepod (1–10 µg total biomass/individual) or amphipod was needed to evaluate ecdysteroid distribution in these organisms. In this study, both *A. tenuiremis* and *L. plumulosus* demonstrated similar patterns in ecdysteroid distribution. Particularly, embryonic ecdysteroids were present in early Stage-I embryos and increased with advancing embryonic development. This pattern supports other observations that generation of an embryonic Y-organ can supplement maternally derived ecdysteroids (Chang, 1992; Spindler et al., 1987), although a Y-organ has not yet been detected in copepods.

Distinct species differences in distribution of 20E were seen in gravid females. In *L. plumulosus*, gravid ecdysteroid concentrations were maximal in females that had recently extruded embryos. Conversely, the maximal 20E titer in gravid *A. tenuiremis* occurred in pre-hatch females. This difference may be explained by variations

in mating behavior and life-cycles between species. For example, these highly prolific copepods will extrude a new clutch within hours of embryo hatching. Consequently, elevated 20E levels in pre-hatch copepod females are likely preparatory for vitellogenesis and ovogenesis of the next brood. This explanation seems plausible since a relationship between elevated ecdysteroids and vitellogenin synthesis has been observed in amphipod (*Orchestia gammarellus*) and isopod (*Porcellio dilatatus* and *Armadillidium vulgare*) species (Blanchet-Tournier, 1982; Gohar and Souty, 1984; Meusy et al., 1977; Souty et al., 1982; Suzuki et al., 1996). In addition, unlike amphipods, isopods, and most macrocrustaceans, copepods cease molting following metamorphosis out of the 5th copepodite stage, suggesting that a Y-organ (or equivalent) is no longer physiologically needed and thus may undergo degradation and loss (Chaix et al., 1976; Charmantier and Trilles, 1979; Lachaise et al., 1981). Subsequently, ecdysteroid synthesis may then occur primarily in reproductive tissues of the now mature adult female (Lachaise et al., 1981). In contrast, *L. plumulosus* undergoes repeated molting throughout its life-cycle, suggesting an enhanced ecdysteroid regulation mechanism which may necessitate an increased reproductive 20E titer mainly during vitellogenesis and egg/embryo extrusion.

Another curious observation in this study was the presence of relatively high ecdysteroid concentrations in males of both model crustaceans. High 20E can be somewhat explained for amphipods which undergo lifetime molting and thus exhibit lifetime ecdysteroid synthesis. For example, during the molt cycle of the amphipod *Orchestia cavimana*, minimal ecdysteroid levels (6.5 pg/mg) occur immediately after ecdysis, and peak levels (295 pg/mg) occur just prior to molting (Graf and Delbecque, 1987). In our study, a high degree of male 20E variability would be expected since ecdysteroids were measured in randomly collected adult amphipods, with molt cycles in various unknown stages of progression. However, male *L. plumulosus* 20E levels were consistent and our data do not support this expectation. In contrast, male copepods cease molting upon sexual maturity and would be expected to have little use for a “molting hormone.” Yet, levels in mature

Table 2  
Occurrence of ecdysteroids in selected micro- and macro-crustaceans

Type	Species/source	Ecdysteroid content <sup>a,b,c</sup>	Method of analysis <sup>d</sup>	Reference
Copepoda	<i>A. tenuiremis</i>		EIA	This study
	Adult female	83 ± 40 (new gravid), 187 ± 121 pg/copepod (old gravid)		
	Adult female	71 ± 26 pg/copepod (non-gravid)		
	Adult male	129 ± 60 pg/copepod		
	Copepodite	79 ± 29 (C1), 115 ± 63 (C4 male), 96 ± 26 (C4 female) pg/copepod		
	Nauplius	219 ± 82 pg/nauplius		
	Embryo	9 ± 5 (newly extruded), 17 ± 7 (pre-hatch) pg/embryo		
	<i>Stenhetia bifida</i>		EIA	This study
	Adult female	58 ± 45 pg/copepod		
	<i>Enhydrosoma propinquum</i>		EIA	This study
Adult female	76 ± 42 pg/copepod			
<i>Microarthridion littorale</i>		EIA	This study	
Adult female	74 ± 30 pg/copepod			
<i>Nannopus palustris</i>		EIA	This study	
Adult female	70 ± 32 pg/copepod			
Cladocera	<i>Daphnia magna</i>		EIA, RIA	Bodar et al. (1990), Mu and LeBlanc (2002)
	Adult female	210 pg/mg		
	Embryo	48.8 ± 13.1 pg/embryo		
	Neonate	28.7 ± 7.0 pg/neonate		
Amphipoda	<i>Orchestia cavamina</i>		RIA	Graf and Delbecque (1987)
	Adult male	13.8 ± 1.4 (A), 6.5 ± 1.8 (C), 295 ± 25 (D1), 67 ± 10 pg/mg (D2)		
	<i>Orchestia gammarellus</i>		HPLC, RIA	Blanchet et al. (1976, 1979)
	Adult female	8–11 (A, B), 8–20 (C), 11–31 (D1), 60 pg/mg (D2)		
	Adult male	14 (A), 50 (B), 12–21(C), 32 (D1), 63 pg/mg (D2)		
	<i>L. plumulosus</i>		EIA	This study
Adult female	133 ± 40 (new gravid), 70 ± 20 pg/amphipod (old gravid)			
Adult female	121 ± 66 pg/amphipod (non-gravid)			
Adult male	168 ± 48 pg/amphipod			
Embryo	5 ± 2 (newly extruded), 21 ± 16 (pre-hatch) pg/embryo			
Isopoda	<i>Oniscus asellus</i>		RIA	Steel and Vafopoulou (1998)
	Adult female			
	Non-breeding	~10 (A, B), 1–10 (C), ~25 (D0), ~75 (D1), ~350 pg/μl (D2)*		
	Breeding	~25 (A, B), ~10 (C), ~20 (D0), ~100 (D1), ~425 pg/μl (D2)*		
	Ovary	1–100 (A, B), ~25 (C), ~20 (D0), ~100 (D1), ~250 pg/mg (D2)		
	<i>Armadillidium vulgare</i>		HPLC, RIA	Suzuki et al. (1996)
	Ovary	~20 (C), ~60 (D0), ~200 (D1), ~90 pg/mg (D2)		
	<i>Helleria brevicornis</i>		RIA	Hoarau and Hirn (1978)
	Adult female	~1500 (A, B), 525 (C), ~3000 pg/mg (D)		
	Adult male	~850 (A, B), 240 (C), ~1250 pg/mg (D)		
<i>Sphaeroma serratum</i>		GC	Charmantier et al. (1977)	
Adult male	1–40 pg/mg			

<sup>a</sup> All measurements from whole tissue homogenates except where noted (\*).

<sup>b</sup> Measurements denote actual ecdysteroid levels except where approximated (~).

<sup>c</sup> Molt stage indicators (A, B, C, D) correspond to the classic identification system by Drach and Tchernigovtzeff (1967).

<sup>d</sup> EIA—enzyme immunoassay; RIA—radioimmunoassay; HPLC—high pressure liquid chromatography; and GC—gas chromatography.

males were similar to levels in immature male C4 copepodites and adult females. One explanation for a high mature male titer is the potential involvement of ec-

dysteroids in spermatogenesis. Studies conducted in the prawn *Macrobrachium rosenbergii* (Sagi et al., 1991) suggest that ecdysteroids may directly or indirectly

participate in regulation of spermatogenesis, possibly by activation of the crustacean androgenic gland (Chang, 1997; Laufer and Landau, 1991). Presently, the androgenic gland has been identified only in malacostracans (Charniaux-Cotton and Payen, 1988).

In the field-collected harpacticoid copepods, Stage-I gravid females generally possessed ecdysteroid titers similar to Stage-I gravid *A. tenuiremis* in spite of highly variable brood sizes among copepod species. Additionally, the ecdysteroid content of whole egg sacs were remarkably similar for all species, regardless of brood size. However, after egg sac ecdysteroids were normalized against brood size, embryos from copepod species producing smaller broods possessed higher ecdysteroid levels than in embryos from species generating large broods. From these data, the uniform ecdysteroid levels observed in Stage-I copepod egg sacs suggest that reproductive females produce similar quantities of ecdysteroids for brood size independent distribution into their embryos. Thus, the highly variable brood sizes reported for these harpacticoid species effect ecdysteroid distribution to individual embryos leading to the observed inverse relationship between ecdysteroid content and clutch size.

In conclusion, our data demonstrate that micro- and macro-crustacean ecdysteroid endocrinology has many similarities. However, several distinctions have been described which indicate that decapod crustaceans and other macrofauna may not be fully comparative models for evaluation of meiofaunal endocrinology. Unfortunately, few studies previous to ours are available regarding any endocrine functions in meiofaunal-sized organisms other than cladocerans. In cladocerans, embryonic 20E content ( $49 \pm 13$  pg/embryo) was greater than ecdysteroid levels detected in *A. tenuiremis* embryos, while neonatal 20E ( $29 \pm 7$  pg/neonate) was less than ecdysteroid levels measured in *A. tenuiremis* nauplii (Mu and LeBlanc, 2002). 20E has also been quantified during the molting and reproduction of some isopod, and amphipod species other than *L. plumulosus* (Table 2). Thus, our future studies will use this fluorogenic EIA assay to further compare ecdysteroid concentrations and distributions in additional meiofaunal-sized taxa, including nematodes, in order to assess relative degrees of endocrine homology. Additionally, as *L. plumulosus* and *A. tenuiremis* are commonly used estuarine bioassay organisms, these approaches are also being applied to investigate endocrine and hormonal modulations induced by reproductive and developmentally toxic chemicals, such as endocrine disruptors, pesticides, and polynuclear aromatic hydrocarbons.

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