

Evidence for the existence of juvenile hormone in the horseshoe crab

by

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ABSTRACT

Lipid-based hormones known as the juvenile hormones (JH) are ubiquitous among the arthropods, but their presence, functions, and sites of production in the horseshoe crab, *Limulus polyphemus*, remain unknown. Large size and lack of secondary sex characteristics in adult female horseshoe crabs may indicate continuous growth and molting throughout life, which is the outcome of high JH levels in insects and crustaceans. Here a study was undertaken to detect and localize lipid-based hormones in horseshoe crab hemolymph and tissue. Capillary electrophoresis and RP-HPLC analyses indicate the presence of a JH-like compound in subadult horseshoe crab hemolymph. The compound is present only in much lower amounts in the hemolymph of adult male and adult female horseshoe crabs. Identification of this compound was based on its similar retention time to standard JH, co-migration with added JH, and cross-reactivity with a polyclonal antibody to JH III. In addition, immunohistochemistry was used to localize the production site of this compound. Analysis of neural tissue, the assumed site of production, yielded no reactivity with labeled anti-JH III antiserum. In larval animals, however, reactivity was noted in yolk contained within the digestive tract. Since the larvae are lecithotrophic and feeding only on their yolk reserves, JH in the gut may be maternal, deposited in the egg before laying. Based on these results, we conclude that horseshoe crabs produce a lipid-based, JH-like hormone, with functional similarity to JH III in insects (i.e., maintenance of the juvenile form during growth and molting.) This paper is the

first substantiation of such a hormone in horseshoe crabs. Our findings suggest that JH will be found in other chelicerates as well.

INTRODUCTION

Molting, Maturation, and Sexual Development in *Limulus*

The Atlantic horseshoe crab, *Limulus polyphemus*, has a life cycle similar to that of other arthropods, in that growth during its juvenile stages of life necessitates periodic molting. In males, a terminal molt occurs upon reaching sexual maturity, whereupon the secondary male characteristics are gained. One important marker of a male's maturity is the appearance of "claspers" (Fig 1)—a modification of the pedipalps. Their transformation from a weak chelate form (Fig. 3) to a robust hooked claw allows males to grasp females during amplexus. The other major characteristic of a sexually mature male is a concavity in the frontal prosoma (Fig 2), also facilitating amplexus by allowing the male to rest the front rim of its shell on the convex opisthosoma (rear segment) of the female.



Fig 1. Pedipalps from a mature male (left) and female (right) horseshoe crab.



Fig. 2. Comparison of carapace shape in mature male (top) and female (bottom) horseshoe crabs. Note the prosomal arch in the male.

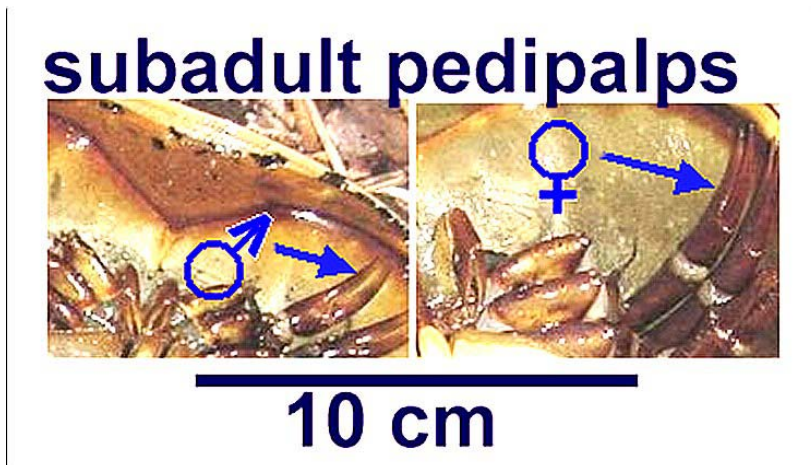


Fig 3. Pedipalps from subadult male and female horseshoe crabs.

Females show no evidence of such secondary sex characteristics.

Superficially, an adult female closely resembles a subadult of either sex, except for its larger size. Female horseshoe crabs grow significantly larger than males (Suggs et al 2002). In Fig 4, the large animal is the only female; this is a typical example of the size difference between the sexes. There is apparently no set size at which maturity is attained in the female, since mature (i.e., egg-producing) females can be found in a large range of sizes (Suggs et al 2002.)



Fig. 4. An example of the typical size difference between mature female (front-most animal) and male (all others) horseshoe crabs.

Positive gender identification in either a female or a subadult requires examination of the gonadal pores (Fig. 5.) These pores—the outer orifices of the sperm- or egg-releasing ducts—are located on the posterior surface of the flap covering the book gills. In males, the pores are conical, and each of the paired structures terminates in a small round opening. In females they are flat ovals opening via horizontal slits, consistent with their eventual role in shedding large (1-2mm) gametes. Adult males produce sperm year-round and will usually shed some whenever the pores are touched. In adult females, the color of the flap bearing the pores usually corresponds to the animal's reproductive status. An olive color generally indicates a gravid animal, while in those not producing eggs (either due to immaturity or seasonal variation), the color is yellow. A gravid female will release eggs if the ducts leading to the pores are stimulated.



Fig. 5. Female (left) and male (right) gonadal pores.

The very large size attained by some female horseshoe crabs, coupled with the lack of secondary sex characteristics, may indicate the absence of a terminal molt in females. Juvenile hormones typically keep arthropods from metamorphosing. Neither diapause nor metamorphosis (other than the appearance of male claspers) occurs in *Limulus*: the persistence of a juvenilizing hormone could promote continued growth after sexual maturity is reached.

While much is known about the neuroendocrine control of molting and maturation in relatively recently diverged taxa of arthropods (insects and crustaceans), information on these processes in the more ancient *Limulus* is lacking. The specific hormones that regulate the molting process and which underlie the sex-related differences in maturation remain largely undocumented. Given the ubiquitous nature of the juvenile hormones among the arthropods, it is likely that horseshoe crabs share similar hormonal pathways for maturation as insects and crustaceans. However, such pathways could be more recent evolutionary developments. The development of neuropeptides to regulate production of sesquiterpenoid hormones (e.g, juvenile hormone) appears to have occurred several distinct times in arthropod evolution (reviewed by Tobe & Bendena 1999). Thus, presence of a similar, lipid-based hormone in horseshoe crabs does not necessarily indicate an ancient evolutionary origin of insect JH.

Horseshoe crab stocks are currently being threatened by overcollection for bait (Germano 2003, Clines 2000, McCartney 2000), and most of the information about maturation in *Limulus* (e.g., how long it takes to reach sexual maturity, how long it lives, how to determine age) is anecdotal (e.g., Grady et al 2001,

Sekiguchi et al 1988.) A study of neuroendocrine control of growth and maturation in *Limulus* is essential to the management of this animal resource. In addition, determining if agricultural mimics of molting hormones and juvenile hormones will affect horseshoe crabs requires knowing the roles of these hormones at all stages of the life cycle. Not even the mechanism of sex determination is known in these oldest of arthropods; neuroendocrine studies may provide some answers to this problem as well.

Juvenile Hormones in the arthropods

A1. Functions in insects

Many of the hormones that are involved in arthropod maturation are lipid-derived (juvenile hormone, ecdysone, and the steroid sex hormones.) The presence, functions, and sites of production of these hormones in insects and crustaceans are well known. Particularly notable among these hormones is the class of sesquiterpenoid molecules known as the juvenile hormones (JH; Fig. 6A), whose crucial functions in insect ecdysis were discovered in 1934 (Wigglesworth 1934.) While the steroid hormone ecdysone is responsible for triggering an insect molt, JH is responsible for determining the outcome of the molt. High levels of circulating JH in insect hemolymph at the time of a molt result in a larger juvenile form, while low levels will cause the insect to undergo metamorphosis into a pupa or adult form (reviewed by Laufer & Borst 1983). This "juvenilizing" effect of JH has been experimentally verified in *Rhodinus prolixus*, a true bug that, like *Limulus*, does not undergo a dramatic

metamorphosis (Wigglesworth 1934, 1936, 1940.) Larval *Rhodinus* that were decapitated at critical periods (thereby removing the corpora allata, the site of JH production in the insect brain) developed prematurely into abnormally small adults. Conversely, telebiotic and parabiotic union of decapitated late-instar larvae with early-instar larvae caused the former animals to undergo extra molts into giant nymphs, rather than into adults.

Besides its role in maturation and morphogenesis, juvenile hormone has other functions in insects as well. In fact, evolutionary data indicate that JH's adult reproductive functions probably preceded its role in juvenile morphogenesis (Tobe & Bendena 1999, Sehnaal et al 1996.) JH plays a variety of roles in stimulating reproductive processes and behavior in adult insects. In general, adult female insects show a greater reliance on JH's effects than do adult males (Rose et al 2001), and the list of apparent effects of JH in this group is long. JH appears to be involved in the synthesis and uptake of vitellogenin in adult female insects, and circulating levels of the hormone vary according to the oviposition cycle (Borst et al 2000, Wyatt & Davey 1996.) This effect is exerted both by JH-induced stimulation transcription of vitellogenin genes (Glinka and Wyatt 1996) as well as by interaction of JH with ovarian follicle cell receptors (Davey et al 1993.) JH levels in many insect species are directly correlated with ovarian mass and stage of ovarian development (Koeppe et al 1985, Okuda and Chinzei 1988.) Behavioral and social effects of JH have been noted as well -- a JH-mediated process of worker/queen caste determination and division of labor in honeybees, for example, has been experimentally well established (Sullivan et al 2000,

Cnaani et al 2000, Bloch et al 2000, reviewed in Robinson & Vargo, 1997.) JH is also necessary for stimulating the muscle development required for oviposition (Rose et al 2001.) In male insects, a few instances of JH-modulated behavior patterns have been noted. Corpora allata removal from adult male locusts triggers a cessation of mating behavior (Pener 1965, Pener 1967) and in the black cutworm, JH is involved in the behavioral response to female pheromones (Gadenne et al 1993.)

JH may have non-reproductive functions as well. In crickets, for example, JH is involved in the stimulation of persistent neurogenesis (Cayre et al 1994.)

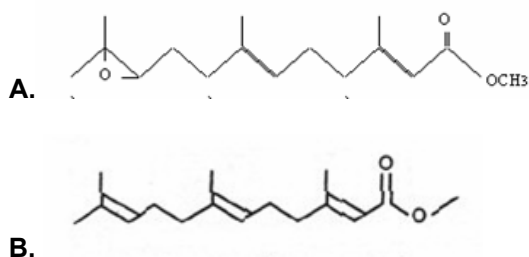


Fig. 6. Structures of (A) juvenile hormone III (from insects), (B) methyl farnesoate (from crustaceans.)

A2. Functions in crustaceans

An unepoxidated form of JH III, methyl farnesoate (MF; Fig 6B), is produced in the mandibular organ of crustaceans (Laufer et al 1987). The functions of MF appear to be similar to those of JH in insects; i.e., control of morphogenesis in juveniles and regulation of reproduction in adults. Spider crab (*Libinia emarginata*) juveniles with high levels of circulating MF molt into small-

claw males while low MF levels produce large-claw males, an observation consistent with the known "juvenilizing" function of the similar hormone in insects (Rotllant et al 2000.) Barnacles (*Balanus amphitrite*) exposed to physiological concentrations of MF sometimes fail to settle and metamorphose, while high (non-physiological) concentrations trigger a premature and often incomplete molt. The prior effect is also consistent with JH's juvenilizing function, while the latter effect indicates an interaction of MF with ecdysone. Ecdysone alone is known to trigger incomplete molting in both insects and crustaceans (Smith et al 2000). Furthermore, ecdysone causes premature molting of horseshoe crab larvae (Jegla & Costlow 1979) and unsuccessful molting (i.e., death before completion) in juvenile horseshoe crabs (Flynn 1997, Dr. Gibson, personal communication.) A similar result, further demonstrating the juvenilizing function of MF, has been observed in prawns (*Macrobrachium rosenbergii*), which exhibit a dose-dependent delay of growth and metamorphosis in response to ingestion of MF-enriched *Artemia* nauplii (Abdu et al 1998.)

Evidence for a gonadotrophic function of MF in crustaceans has been obtained for several species. Circulating MF levels in female crabs (*Cancer pagurus*) are highest during periods of vitellogenesis (Wainwright et al 1996). Also, cultured spider crab mandibular organ tissue from females undergoing vitellogenesis display higher *in vitro* MF secretion than mandibular organ tissue (the MF-producing organ) from non-oocyte-producing females (Laufer et al 1987). Additional evidence for MF as a stimulant of oocyte production has been

obtained from studies indicating enhanced fertility in MF-treated shrimp (Laufer et al 1997) and increased ovary size in MF-fed crayfish (Laufer et al 1998.)

As with JH, MF probably plays a role in reproductive behavior as well. Circulating MF level in male spider crabs, for example, is strongly correlated with observed levels of mating behavior (Sagi et al 1994.)

MF may also have roles unrelated to reproduction and development. At least one study (Lovett et al 1997) has indicated that stressor conditions such as handling, disease, or poor environmental conditions raise circulating MF levels in green crabs (*Carcinus maenus*.)

The presence of JH in arthropod taxa other than Insecta and Crustacea remains unresolved. In 1984, Dorn stated that “the presence of JH or JH-like compounds has never been demonstrated in Chelicerata by physiochemical methods or bioassays to my knowledge.” Since this statement was made, no new verification has come to light. Some researchers have shown indirect indications that ticks may produce JH, although the hormone's presence in ticks has not been established unequivocally. In 1979, the topical application of JH analogs to tick eggs was shown to block embryogenesis (Pound & Oliver 1979). More recently, ticks have been shown to produce JH-binding proteins (Lomas et al 1996), thus pointing to a likely endogenous production of JH or a similar hormone. Injection of ticks with JH, however, has failed to stimulate vitellogenin synthesis (Chinzei et al 1991), an observation that may indicate either a lack of JH production or different roles for the hormone if it is produced. Arachnids represent a taxonomic class that is distinct from the Merostomates (horseshoe

crabs and extinct sea scorpions), but both are in the same jawless subphylum, the Chelicerata (see Fig 7 for a phylogenetic tree of the Arthropoda; Clowes 2003.) JH is well-studied in the mandibulate arthropods (insects and crustaceans), but its existence in the chelicerates should not be extrapolated from such studies. The studies showing that ticks bind JH and cannot produce embryos in its presence are so far the most compelling evidence that chelicerates may produce this hormone.

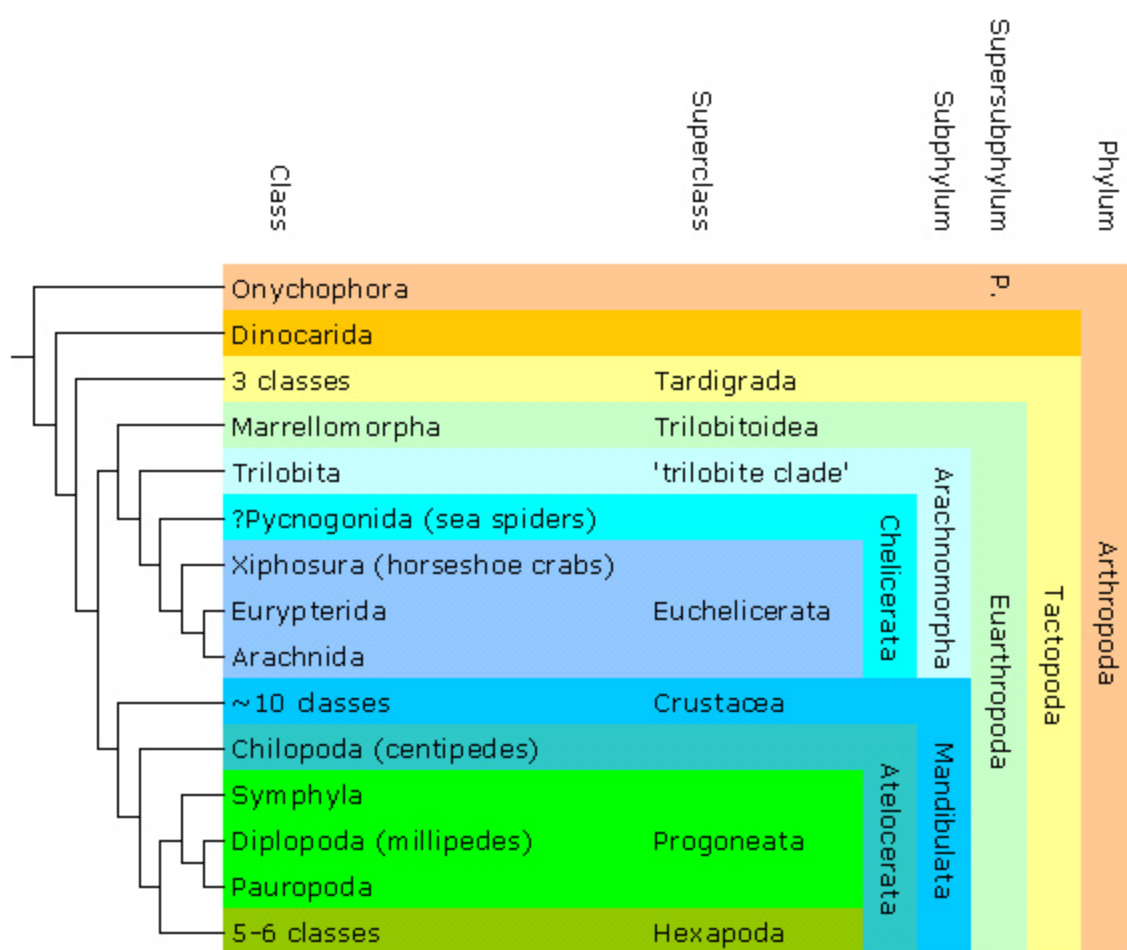


Fig.7. Phylogeny of the arthropods (Clowes 2003.) Insects are part of the Hexapoda; horseshoe crabs and arachnida are in the Euchelicerata.

No direct evidence yet exists for the presence or absence of JH or similar hormones in horseshoe crabs. One series of experiments has shown that JH and similar compounds exert no effect on embryonic and larval development in *Limulus*, or (at high concentrations) a toxic effect leading to abnormal dorsal organ development (Jegla 1982). These results do not disprove the existence of JH in horseshoe crabs—if JH plays a similar role in these animals as in insects, then JH levels in embryos and larvae would already be quite high. Thus, the additional exogenous application of the hormone would show no additional effect, until concentrations became toxic.

The pattern of molting/maturation in female horseshoe crabs (continued growth with apparent lack of a terminal molt) is consistent with the "juvenilizing" function of JH/MF that has been noted in both insects and crustaceans. This observation, coupled with the widespread existence of the JHs among the arthropods, indicates that it is both possible and likely that lipid-derived hormone similar to JH is responsible for instar growth in *Limulus*.

B. Sites of production

JH III is produced in insects by the corpora allata, a pair of neuroendocrine organs projecting from the brain. Attached to the corpora allata are the corpora cardiaca, which are involved in the production of allostatin and allotropin. Both of these hormones likely play a role in the regulation of JH. Ecdysone, the "molting hormone" whose production triggers molting in nearly all arthropods, is produced by the prothoracic gland in insects when stimulated by ecdysiotrophic hormone

from the brain. (See Fig. 8 for a schematic diagram of the insect neuroendocrine system)

In crustaceans, several hormones are produced in the eyestalks. Various regulatory hormones including molt-inhibiting hormone (an inhibitor of ecdysone) and mandibular-organ-inhibiting hormone (an inhibitor of MF) are produced by the X-organ, located in the eyestalks (see Fig. 9.) Ecdysone itself is produced by the Y-organ, located at the base of the antennae, while the mandibular organ produces MF. Although the locations of these neuroendocrine organs differ from the locations of their analogous counterparts in insects, the two systems are thought to be analogous.

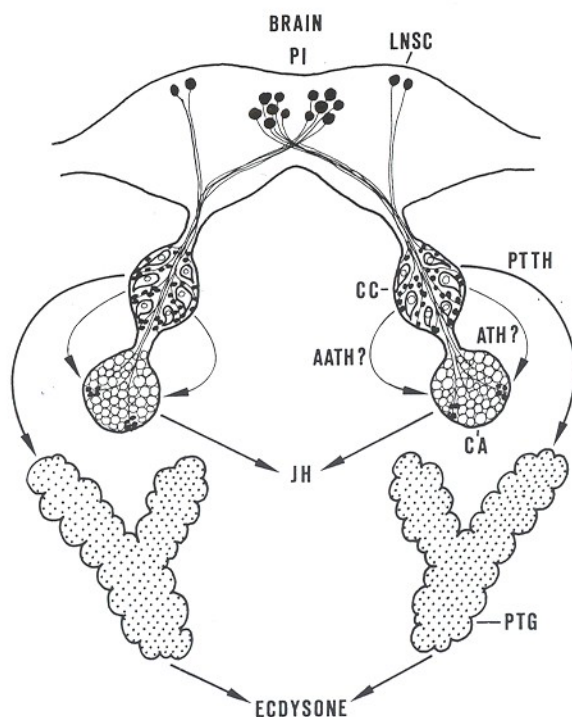


Fig. 8. Insect neuroendocrine system. (Dorn 1984.) AATH = antiallotropic hormone; ATH = allotropic hormone; CA = corpus allatum; CC = corpus cardiacum; JH = juvenile hormone; LNSC = lateral neurosecretory cells; PI = pars intercerebralis; PTG = prothoracic gland; PTTH = prothoracicotrophic hormone.

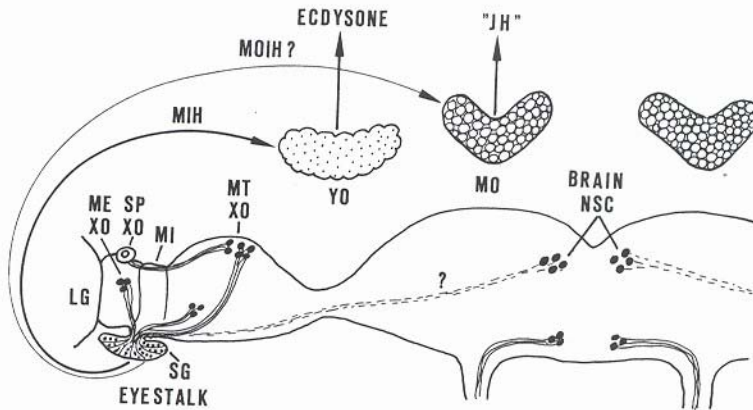


Fig. 9. Crustacean neuroendocrine system (Dorn 1984.) "JH" = methyl farnesoate, LG= lamina ganglionaris; ME XO = X-organ of medulla externa; MI = medulla interna; MIH = molt-inhibiting hormone; MO = mandibular organ; MOIH = mandibular-organ-inhibiting hormone; MT XO = X-organ of medulla terminalis; MSC = neurosecretory cells; SG = sinus gland; SP XO = sense pore X-organ; YO = Y-organ.

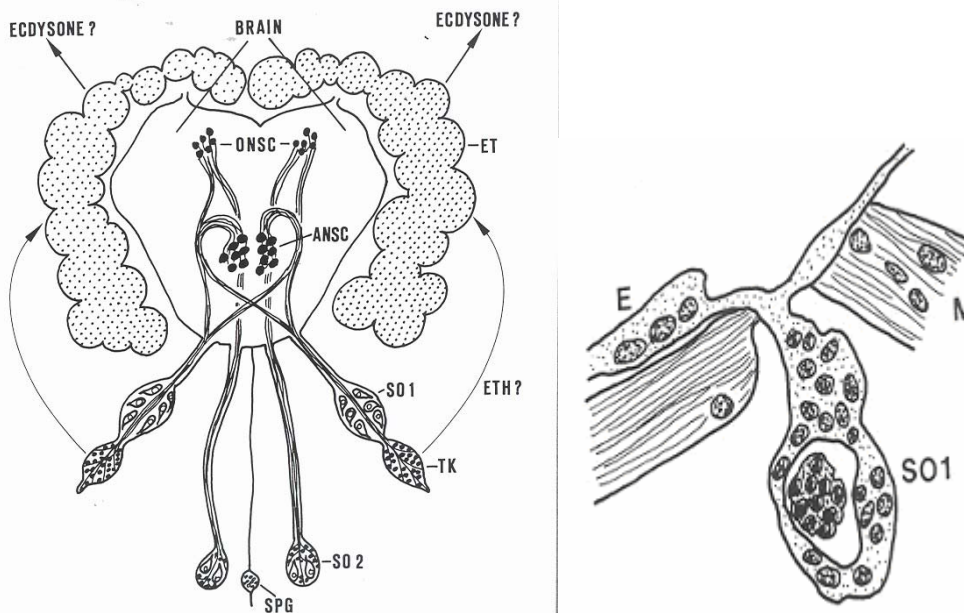


Fig. 10. Araneae (spider) neuroendocrine system, left; diagram of embryonic development of Schneider's organ I in a spider embryo, right (Dorn 1984.) ANSC = aboral neurosecretory cells; ET = "endocrine tissue"; ETH = "endocrine tissue" stimulating hormone; ONSC = oral neurosecretory cells; SO 1, 2 = Schneider's organ I and II; SPG = suprapharyngeal ganglion; TK = Tropfenkomplex; E = ectoderm; M = muscle; SO1 = differentiating anlage of Schneider's organ I.

Horseshoe crabs' neuroendocrine systems are not similar to either that of the insects or of the arthropods. They lack eyestalks and mandibles (thus no mandibular organ), and have no homologs of the corpora allata and prothoracic glands; consequently the sites of production of the relevant hormones in these animals must differ from those of other arthropods. Some researchers believe that Schneider's organ I (a part of the neuroendocrine system in spiders) is the analogous organ to the insect corpus allatum (Dorn 1984; see fig 10.) This conclusion is based on the similarity in embryonic differentiation in the two organs; there is no evidence that Schneider's organ makes any hormones.

C. Methods of Detection and Localization

Juvenile hormone detection via radioimmunoassay is a well-established technique, using rabbit antiserum to insect JH III (Goodman et al 1995.) In addition, detection of JH or related hormones has also been accomplished via HPLC (Range et al 2002) and GC/MS methods (Baker et al 1984.)

Capillary Electrophoresis

Capillary electrophoresis (CE) is a relatively new method (developed in the late 1980s; see Kemp 1998 for a review) of chemical separation and analysis. Its applications are similar to those of HPLC; however, CE poses several distinct advantages over HPLC, most notably smaller required volumes of analyte and buffer, as well as improved sensitivity. Like other forms of electrophoresis, CE operates by exploiting the differing migration rates of molecules in an electric

field based on their size and charge. In CE the stationary phase is a buffer solution inside a narrow fused-silica capillary. The exposed silanol groups on the interior surface of the capillary are negatively charged and attract hydrogen ions in the solution, thereby creating a flow (called bulk flow or endo-osmotic flow) through the capillary toward the cathode. Because of this endo-osmotic flow, even negatively charged molecules will migrate in the direction of the cathode, although migration will be slower than for positively charged molecules.

Although CE is most commonly used for the separation of proteins, peptides, nucleic acids, and small charged molecules, its use for lipid analysis has also been established (for examples, see Bohlin et al 2003, de Oliveira et al 2001, Collet & Gareil 1996). For effective separation of neutral lipophilic molecules, a surfactant is usually added to the run buffer. The presence of the surfactant permits the formation of micelles with a hydrophobic interior and charged exterior. Rate of migration is determined by the strength of the hydrophobic interaction of the neutral analyte molecules with the micelles. In addition to a surfactant, another common addition to CE buffer is cyclodextrin (CD), a cup-shaped assembly of glucose units that is water-soluble with a hydrophobic interior. CDs are often used to separate chiral enantiomers (Rudzinska et al 2002) and also to improve resolution of lipophilic analytes, in the event that the analyte migrates at the same rate as the surfactant (Nishi and Matsuo 1991). In addition, acetonitrile is often used in the buffer when analyzing lipids.

In this project a protocol for indirect detection of fatty acids was followed (de Oliveria et al 2001) using a buffer with sodium dodecylbenzene sulfonate (SDBS) as surfactant and chromophore and dimethyl β -cyclodextrin for solubilization and further enhancement of separation, plus 35% acetonitrile.

Although CE has many advantages as an analysis technique, it also evidences a variety of problems. CE analyses are especially susceptible to molecular-weight-independent migration—i.e., a shifting of retention times caused by contaminants, capillary blockage, changes in buffer composition, and other factors. This type of problem can make it difficult to definitively identify a peak. CE's high sensitivity can also lead to detection of contaminants, causing poor reproducibility of data. In addition, detection of lipids or lipid-like molecules in CE is not as well-established as detection of proteins, and such analyses require (as described) a micellar system for proper separation. Introduction of the micelles into the capillary complicates the analysis, necessitating further refinement to ensure reproducibility and to prevent capillary clogging. The type and concentration of surfactant used, the pH of the buffer, and the presence of flow modifiers and other additions can affect the micelle migration and both the micelle-analyte and micelle-capillary interactions.

HPLC

High performance liquid chromatography (HPLC) is a more well-established analysis and separation technique. Several investigators have performed successful detection of both JH and MF in biological tissue using

HPLC. One early attempt (Borst & Tsukimura 1991) achieved a lower detection limit of 250pg/mL for the detection of MF in the hemolymph of lobsters, green crabs, and spider crabs using normal-phase HPLC. In this technique, the stationary phase is polar while the mobile phase is nonpolar. These authors do note that reversed-phase (RP-) HPLC, the method utilized here (using a polar mobile phase and nonpolar stationary phase), showed a precedent of much lower sensitivity, achieving detection of MF only when physiological levels exceeded 75 ng/mL (Bruning et al 1985.)

Range et al (2002) used RP-HPLC to detect several forms of JH and JH diol in hemolymph of the armyworm *Spodoptera frugiperda*, although this study does not indicate the concentration limits of detection. A modified form of these authors' protocol (with changes as noted in the methods section) was used here.

Immunohistochemistry

Immunohistochemistry (both whole-mount and electron microscopy) is a valuable method for localization of target molecules in fixed tissue. Although a rabbit antiserum to insect JH III was available for use in this project, the lipid nature of the target molecule made many fixation procedures questionable. Since tissue fixation usually involves extensive alcohol dehydration, fatty-acid crosslinkers, and often surfactants (such as Triton-X), there is a strong possibility that a lipophilic molecule such as JH would be extracted or would lose antigenicity during the fixation process. Lack of success in some

immunomicroscopy attempts for this project could have been due to these treatments.

JH analogs

A wide variety of JH analogs exist which show potent biological activity in the arthropods. Such products are useful as insecticides since they disrupt embryogenesis and metamorphosis in larval insects and thereby prevent larvae from maturing to reproductive age.

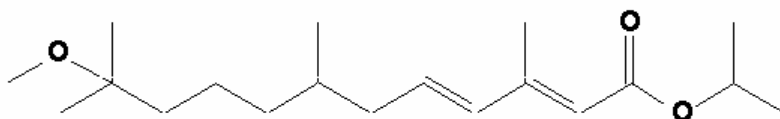


Fig. 11. Methoprene, a synthetic JH analog used as an insecticide.

Methoprene (fig 11) is the JH analog used in this study. This compound has an extensive history of insecticidal use. Methoprene-exposed insect larvae grow abnormally large and/or fail to pupate (Kostyukovsky et al 2000); in exposed pupae, adult emergence is delayed or prevented (Nayar et al 2002.) Such effects are consistent with the known effects of high levels of physiological JH. Methoprene also displays JH-like effects upon adult insects, such as the accumulation of JH-dependent gene transcripts upon topical application (Dubrovsky et al 2002, Zhou et al 2002.)

Methoprene's effects are not limited to insects. Due to the similarity of methyl farnesoate to JH, methoprene exposure in crustaceans produces similar outcomes to those observed among insects. MF-like effects of methoprene have been noted in numerous crustacean species, including water fleas (*Daphnia*)

(Ohlmstead & LeBlanc 2001), grass shrimp (*Palaemonetes*) (Wirth et al 2001), brine shrimp (*Artemia*) (Ahl & Brown 1991), and copepods (*Apocyclops*) (Bircher and Ruber 1988.)

Consistent with the tentative evidence that chelicerates may produce a JH-like hormone, methoprene has also demonstrated effects upon ticks (Chinzei et al 1991, Belozarov 2001) and mites (Chauve 1998.) Methoprene and other JH analogs generally do not exhibit effects upon non-JH-producing species, such as frogs (Henrick et al 2002); this is a primary basis for their presumed safety as insecticides. The observed methoprene effect in ticks and mites is further evidence for the possible existence of JH-like compounds in chelicerate arthropods.

SUMMARY AND SPECIFIC AIMS

The juvenile hormones represent an important group of lipid-derived compounds necessary for regulation of arthropod maturation and reproduction. JHs are known to exist in insects (JH I, II, and III) and in crustaceans (methyl farnesoate.) In both these taxa one of their main functions is to block the effects of ecdysone during molting, thereby retaining the juvenile form. Definitive evidence is lacking for the existence of these hormones in the chelicerates, including the horseshoe crab (*Limulus polyphemus*), the most ancient of the extant species of arthropods. *Limulus* displays a sex-dependent pattern of maturation whereby females grow much larger than males and do not obtain a terminal adult morphology. Such a pattern is consistent with the juvenilizing effects of JH and MF in other

arthropods. Thus, it is possible that a similar lipid-derived compound regulates molting and maturation in *Limulus*, promoting the continued molting and retention of juvenile form retention in adult females.

The hypothesis of the present study was twofold: that JH is present in horseshoe crabs, and that adult females do not (counter to anecdotal claims) undergo a terminal molt, because of persistent high JH levels. Males, as indicated by their change in morphology upon terminally molting, experience a drop in JH levels in maturity. At present, any study of neuroendocrine regulation of growth and molting in horseshoe crabs would be a contribution to knowledge of an understudied branch of arthropod physiology.

MATERIALS AND METHODS

Capillary electrophoresis:

A protocol for indirect UV detection of lipids with CE was followed (Range et al 2002).

Run buffer was prepared according to this protocol as follows: 5mmol phosphate buffer with 35%(v/v) acetonitrile (ACN), 4mmol dimethyl- β -cyclodextrin, and 4mmol sodium dodecylbenzene sulfonate (SDBS.) All reagents were purchased from Sigma. The pH of the buffer was adjusted to 7.0 by the addition of sodium hydroxide and/or hydrochloric acid. The buffer was stored at 4°C.

Capillaries used were bare fused silica, 75µm inner diameter, with an effective length of 56cm, and were manufactured by Agilent Technologies, Palo Alto, CA. Analyses were performed in a Hewlett-Packard CE machine with HP's ChemStation software. NaOH solutions and water used for capillary flushing were CE-grade, manufactured by HP. As per the protocol of de Oliveira et al, the voltage was 25kV and the capillary was maintained at 25° C during runs. Sample injection was by pressure (50mbar) for 2 seconds. Detection was at 206nm for most analyses, and also at 224nm for some.

Before use, new capillaries were conditioned by consecutive ten-minute pressure flushes of 1.0M NaOH, 0.1M NaOH, water, and run buffer. Before each run, additional pressure flushes of 0.1M NaOH and of buffer (10 min each) were performed. Buffer vials, including both those used for flushing and the inlet and outlet home vials, were replaced after 3 runs. Initially, at least five replicates of the sample were run from each vial. Later refinements of the technique involved a fresh sample for each run. All solutions, including samples, buffer, water, and NaOH, were filtered through a 0.22µm syringe filter and centrifuged for 5 minutes prior to use.

In order to alleviate problems with capillary clogging that occurred during many analyses, flushing procedure was eventually changed to incorporate a complete reconditioning (as described above, except with 15 minutes per flush) after every run, along with replacement of buffer vials after every run. In addition, sample vials were used for a single run only.

In addition, some other changes to the procedure were performed during later analyses in an attempt to improve reproducibility. Hexane-extracted samples were evaporated to dryness and resuspended in 30% isopropanol/70% run buffer, followed by steam heating to ensure dissolution.

Lipid standards:

Standard solution of JH III was obtained from Sigma (catalog number J2000.) It was diluted 1:100 with isopropanol and stored at -20°C. Prior to analysis it was further diluted to the desired concentration in 30% isopropanol/70% run buffer (v/v) and heated over steam, as described for hemolymph samples below.

Some samples of JH were also subjected to hexane extraction, as described for the hemolymph samples.

Standard solution of 20-hydroxyecdysone was obtained from Sigma, was diluted 1:100 in ethanol and was stored at -20°C. Pre-analysis preparation was identical to that for JH.

Hemolymph preparation:

Horseshoe crabs (*Limulus polyphemus*) were collected in Falmouth, Massachusetts and maintained in tubs of aerated seawater. Tobacco hornworm caterpillars (*Manduca sexta*) were purchased from Carolina Biological Supply and maintained at 27°C on the supplied medium.

Hemolymph samples were drawn from horseshoe crabs with a 20 gauge hypodermic needle inserted either at the carapice hinge or at the articular

membrane of a leg joint. In most cases, the blood ran freely and did not have to be drawn up in a syringe. Control hemolymph samples were obtained from hornworm caterpillars by removing the tail spike with scissors (after topical application of ethanol) and immediately drawing the hemolymph into an Eppendorf pipettor, or letting it drip into a collection tube. JH secretion is highest at mid-instar (except in pre-pupation stage), so blood collection from caterpillars at that stage would have been optimal. To that end, we did not collect blood from quiescent caterpillars, discolored (possibly pre-molt) caterpillars, or caterpillars with molted skin still attached. To enhance the odds that at least some of the hemolymph samples were from mid-instar caterpillars high in JH, we pooled blood samples from several different caterpillars of different stages and sizes (Table 1.)

Length (mm)	Number of animals
38	1
40	2
45	1
50	1
55	3
70	1

Table 1. *Manduca sexta* caterpillars used for blood sample pooling. Approximately 50 -150 μ L of hemolymph was taken from each animal, with the larger volumes from larger animals.

All hemolymph samples (horseshoe crab and caterpillar) were immediately centrifuged for 5 minutes to remove cells and prevent clotting. The retained supernatant of some samples was then stored at -20°C for use in immunological experiments. For CE analysis, the supernatant was mixed with an equal volume of acetonitrile, then centrifuged again for 5 minutes. The resulting supernatant was stored at -20°C .

For CE analysis, stored samples were diluted to the desired concentration in a solution of 30% isopropanol in run buffer. The diluted samples were heated over a steam bath for 5 minutes to ensure complete dissolution. Samples were then mixed with an equal volume of 0.9% NaCl, vortexed and centrifuged for 5 minutes. Half an equal volume of hexane was then added, again followed by vortexing and 5 minutes centrifugation. The hexane layer was retained, and this hexane extraction was repeated with another half-volume of hexane. The hexane fractions were pooled and stored at -20°C if not being used for immediate analysis.

To verify the presence of JH, some hemolymph samples (with acetonitrile) were spiked with JH III prior to hexane extraction and CE analysis. Hemolymph was added to buffer/isopropanol in the desired concentration as described, along with JH at its desired concentration. Steam heating and hexane extraction were then performed as described.

Immunological experiments:

Rabbit antiserum to insect juvenile hormone was obtained from Dr. Walter Goodman at the University of Wisconsin. For CE analysis of blood samples with added antibody, the following procedure was followed. Thawed hemolymph samples (without acetonitrile) were diluted with an equal volume of phosphate-buffered saline (PBS; made from 9 parts 1% NaCl and 1 part 0.1M Sorenson's Phosphate Buffer, pH 7.2-7.4.) JH antiserum was added to a final concentration of 1:10. The tubes were incubated at room temperature on a shaker for at least one hour, then were centrifuged for 5 minutes to remove precipitated proteins.

The supernatant was mixed with an equal volume of acetonitrile and centrifuged for 5 minutes. The supernatant was removed, diluted to the desired concentration in 70% buffer/30% isopropanol, and hexane-extracted, as described above.

To verify activity of the antiserum, the same procedure was used to analyze JH standard plus added anti-JH. In this case, the standard was first diluted 1:100 in isopropanol for storage at -20. The stored JH was then mixed with an equal volume of PBS, followed by the same steps as described above. The hexane extraction procedure was also followed.

HPLC

A previously established protocol for reversed-phase HPLC for detection of JH was used (Range et al 2002), with some modifications as noted. The mobile phase consisted of a linear gradient of 40% - 100% (v/v) acetonitrile in water with a flow rate of 0.2mL / min, and UV absorbance detection at 217nm. The column was a C₁₈, 5µm pore size, dimensions 4.6mm by 150 mm. Analyses were performed at 4° C. All samples were filtered through a 0.2µm syringe filter prior to analysis. The mobile phase was also filtered through a 0.2µm filter before use.

Preparation of standard

JH standard (JH III, Sigma catalog number J2000) was originally prepared at a concentration of 100nmolar in 40% (v/v) acetonitrile in water, and was stored at -20°C. This storage procedure was found to cause degradation over time (Dr. Walter Goodman, U. Wisconsin, personal communication.) Subsequent stock of JH was diluted to 10mg/mL in hexane, divided into aliquots containing 0.01mg JH, and evaporated to dryness. Aliquots were stored dry at -20°C. On the day that HPLC analysis was to be performed, an aliquot was resuspended (with vortexing) in 40% acetonitrile to a concentration of 100µmolar.

Preparation of hemolymph

Hemolymph samples were obtained as described for CE, above. Early HPLC analyses were done with hemolymph samples that had been previously frozen with 50% ACN and then hexane-extracted as previously described, evaporated to dryness and resuspended to the desired concentration in 40% ACN. To rule out the possibility of ACN-induced degradation of endogenous JH, later analyses were performed on hemolymph samples that had been prepared using a modified version of the extraction protocol of Goodman et al (1995). For this process (illustrated in Fig. 12), 100µL of either thawed or fresh hemolymph (which was already centrifuged to remove cells) was added to 100µL ACN and centrifuged for 5 minutes. The supernatant was removed and transferred to a microcentrifuge tube containing 500µL 4% NaCl and 250µL hexane. The pellet was washed twice with 100µL ACN, which was then also added to the brine/hexane mixture. After vortexing and phase separation, the hexane layer

was transferred to a new tube. Another 250 μ L of hexane was added and the extraction was repeated two more times, with all hexane layers ultimately pooled. The hexane extract was evaporated to dryness using a vacuum concentration centrifuge and was stored dry at -20°C. On the same day that the HPLC analysis was to be performed on a sample, the sample was resuspended to the desired volume in 40% ACN.

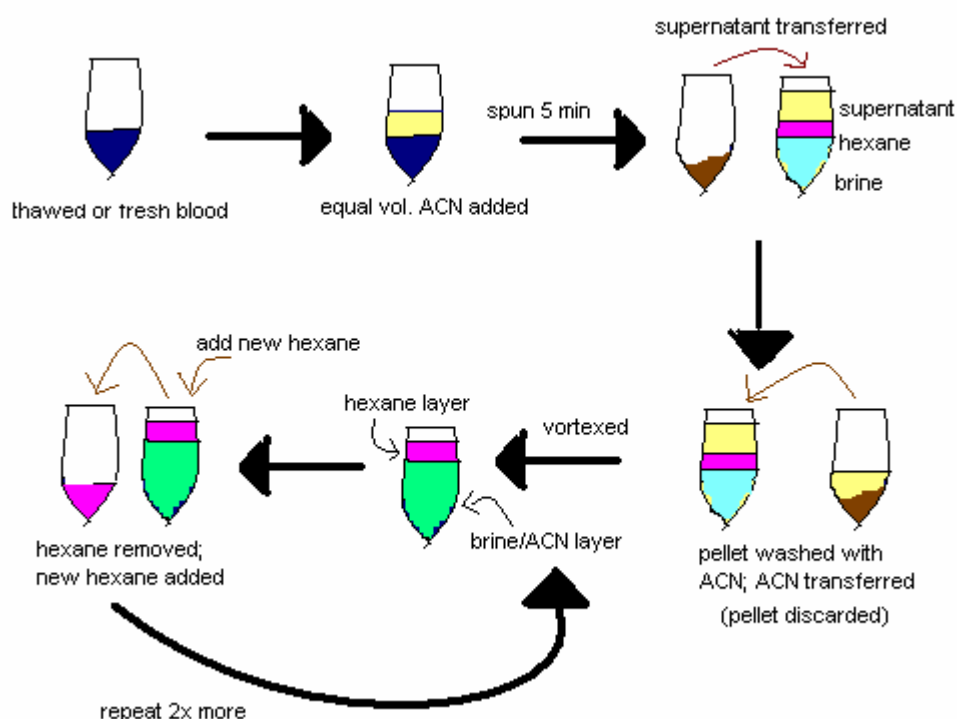


Fig. 12. Pictorial representation of the hexane extraction procedure used here to prepare hemolymph samples for analysis.

To verify the identity of putative JH peaks, some co-injected samples were prepared as follows. After running a hemolymph sample, 250 μ L of the sample was transferred to a new vial, and 250 μ L of JH standard (resuspended as described above) was added to the same vial. This combined sample was then analyzed in HPLC.

Additionally, the cell pellet formed from initial centrifugation of harvested blood was, in some cases, immediately resuspended in pyrogen-free water and vortexed to cause lysis. Aliquots of the lysate (500 μ L, taken from an original 1mL of blood that had been diluted to 7.5mL in water) were hexane-extracted and dried as described.

Immunological experiments:

Addition of anti-JH to thawed hemolymph samples for HPLC was performed as previously described for CE. After the antiserum incubation and the addition of acetonitrile, the supernatant was hexane-extracted as described above for other HPLC samples. Anti-JH was also added to hemolymph samples that had been hexane-extracted and dried immediately after harvest, never having been frozen. In these cases, the dried extract was resuspended in PBS prior to antibody incubation. Following incubation, the previously described extraction procedure was performed.

Immunohistochemistry

A. Wholemounds for light microscopy

Suspecting that JH might be produced in the central nervous system, we prepared wholemounts of nervous system from juvenile horseshoe crabs (15, 20, and 25 mm in length.) For primary fixation, animals were injected with 2% glutaraldehyde in phosphate buffer. Neural tissue removal was performed by cutting the fixed animal longitudinally down the midline (using a razor blade), then

locating and removing the nerve cord, brain, and ganglia. After dissection, tissue was placed in 2% glutaraldehyde overnight, then placed in phosphate buffer 12-24 hrs. Samples were then placed in primary antibody (1:10 in phosphate buffer), 12-24 hours, followed by another 12-24 hr buffer rinse and then 12-24 hrs in secondary antibody (1:50).

Immunostaining of amoebocytes was performed as follows. Circles of 8 mm inner diameter were drawn on microscope slides with a PAP pen (Fig 13). A drop (10-20 μ l) of freshly drawn blood was placed in the center of each circle, and cells were allowed to adhere and spread (5 minutes.) The area was then rinsed with distilled water for 5 minutes to lyse the cells. The water was blotted and replaced with 10% normal goat serum in PBS, which was allowed to sit for 30 min to block non-specific binding sites. The serum was blotted and replaced with 10% anti-JH in PBS, which was allowed to sit for one hour at room temperature. Slides were rinsed three times (20-60 min each) with 10% NGS in PBS, were treated with either FITC-linked or HRP-linked goat anti-rabbit, 10% in PBS. The secondary antibody was left on the slides for one hour at room temperature, then slides were rinsed with PBS three times and left in the final rinse for 1 hour. FITC-treated slides were rinsed with distilled water for 5 minutes, blotted, mounted in glycerin, and viewed with epifluorescence (excitation 490nm, barrier filter 520nm.) HRP-treated slides were blotted to remove PBS and were treated with a 1:10 dilution of metal-enhanced DAB (Pierce) in stable peroxide buffer. After 10-20 minutes, the slides were rinsed with distilled water, allowed to air-dry and coated with immersion oil for viewing.

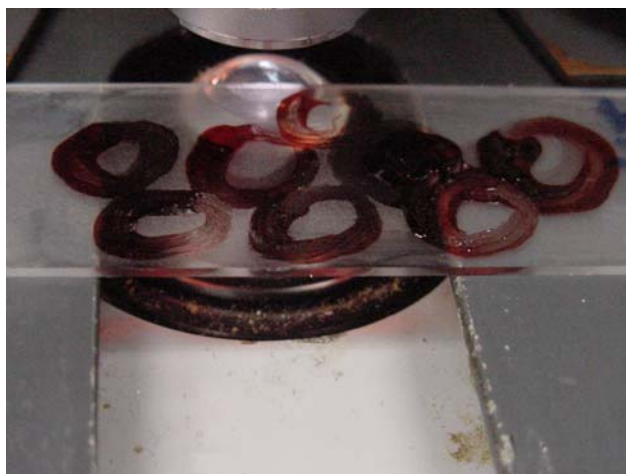


Fig. 13. Hydrophobic wells created on a slide using a PAP pen; used for immunostaining of blood cells.

For immunostaining of eggs, the following procedure was followed. 200 mg (approximately 250 ml) of eggs were added to PBS to make 1 mL. The eggs were crushed, suspended and vortexed, and subdivided into 2 Eppendorf tubes. The homogenate was spun 5 min., decanted, resuspended in 1 mL 10% normal goat serum in PBS, vortexed and leave on a shaker for 20 min. This was repeated three times. Incubation was in 1:10 anti-JH in PBS for one hour on a shaker, vortexing occasionally. After one hour, the homogenate was spun, then rinsed in PBS by resuspending the pellet, vortexing and shaking for >20 minutes. This was repeated three times. After the last spin, the sample was resuspended in FITC-linked or HRP-linked goat anti-rabbit (1:10 in PBS) and incubated on a shaker for 1 hr. with frequent vortexing. Samples were rinsed with PBS three times as before. For FITC-incubated sample, the pellet was spread on a slide, covered in glycerin and a cover slip, and viewed with epifluorescence at 490 nm with 520 nm barrier filter. For the HRP-incubated sample, the pellet was resuspended in 1:10 metal-enhanced DAB in stable

peroxide buffer (Pierce), and incubated with frequent vortexing for >30 minutes. The tube was then spun and observed for color change.

B. Frozen sections for light microscopy

Whole juvenile horseshoe crabs of several sizes were injected with 4% paraformaldehyde in phosphate buffer, and dissected to expose neural tissue, using one of the dissection procedures illustrated in Fig 14. The fixed animals were then placed in 4% paraformaldehyde overnight at 4°C. The samples were washed in phosphate buffer for at least one hour, with several changes of buffer. They were then placed in a 30% (w/v) sucrose solution in deionized water, and left at 4°C for at least 48 hours. Samples were covered with TFM™ Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC) and frozen on dry ice. TFM™ was then used to freeze the samples to a cryostat chuck. Sections of 20µm thick were mounted on slides that had been coated with egg white. Slides were stored at -20°C.

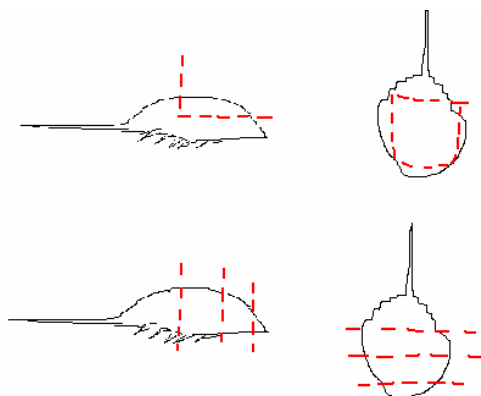


Fig. 14. Dissection techniques used here (left, side view; right, top view.) Dashed lines indicate cuts. Top, removal of dorsal carapace to expose entire nerve cord. Bottom, slicing to separate neural ganglia.

C. Embedded sections (for TEM and light microscopy)

Horseshoe crab larvae and first-stage juveniles were injected with 2.5% glutaraldehyde in phosphate buffer (PB) and dissected using one of the techniques illustrated in Fig 14. Samples were placed in glutaraldehyde for one hour, then rinsed with PB, on a shaker, for 30 minutes. Samples were postfixed in osmium tetroxide (2% in bicarbonate buffer) for one hour, then rinsed three times with deionized water. Dehydration was as follows: 50% ethanol for 10 minutes, 70% ethanol overnight, 90% ethanol 10 minutes, 100% ethanol 3 times at 10 minutes each. Tissues were embedded in Spurr's resin by first infiltrating with 2:1 ethanol/Spurr's, then 1:1, then 1:2, then pure Spurr's, with all steps done for 1-2 hours on a shaker. Samples were placed in molds in a fresh change of resin for 2 hours at room temperature, then cured for at least 24 hours at 60°C.

Post-embedding processing for TEM

For TEM, ultrathin (approximately 100 nm) sections were cut with a diamond knife and were mounted on nickel grids. Following a modified version of the protocol of Brumwell & Martin (1996), grids were floated section-side down on droplets of the solutions subsequently described, for the times indicated. During these processes the grids were kept in covered petri dishes with a moistened paper towel to maintain humidity. Rinse buffers were either 0.1M phosphate buffered saline (PBS), or 0.1M PBS containing 0.05% Tween-80 and 0.25% bovine serum albumin (subsequently referred to as PBS-Tween-BSA.) Before incubation in antibody, as subset of grids was etched with 5% hydrogen

peroxide for 3 minutes. Grids were then given three distilled water rinses (at least 5 minutes each), placed in anti-JH diluted 1:100 in 0.1M PBS-Tween-BSA for 2 hours, rinsed three times (>5 minutes each) with PBS-Tween-BSA, and then placed in 15-nm gold conjugated goat anti-rabbit, diluted 1:50 in PBS-Tween-BSA, for 1 hour. Following this incubation the grids were rinsed three times with PBS-Tween-BSA, then rinsed twice in PBS, and once in distilled water. Grids were then placed in 2% glutaraldehyde in PBS for 10 minutes and rinsed 3 times in distilled water. Metal staining was performed as follows: saturated uranyl acetate in 1:1 ethanol-water for 5 minutes, three 5-minute distilled water rinses, 0.8% lead citrate at pH 12 for 30 seconds, and three more 5-minute distilled water rinses. Before viewing, the grids were dried by blotting with bibulous paper.

Post-Embedding Processing for Light Microscopy

For light microscopy, semithin (approximately 0.5 μm) sections of the same embedded tissue were cut with a diamond knife and were either air-dried or heat-dried (80° on a heat block) onto glass cover slips or Superfrost® (poly-D-lysine coated, Fisher) slides. Following the protocol of Kilman and Marder (1996), the sections were then deresinated by immersion in a solution of 100% ethanol (50mL), propylene oxide (50mL) and saturated sodium hydroxide (5g, not fully dissolved.) Rehydration was then performed by immersing the sections in 100% ethanol, 90% ethanol, 70% ethanol, 50% ethanol, 30% ethanol, and then distilled water (at least 5 minutes each.) Some slides were placed in a

solution of 1% sodium meta-periodate in 0.1M phosphate buffered saline (PBS) for 5 minutes to expose antigenic sites. They were then rinsed in distilled water for 45 minutes and in PBS for 10 minutes. The slides were blocked with 10% normal goat serum in PBS for 1 hour. Antiserum for JH was diluted 1:100 in PBS with 10% bovine serum albumin added, and the slides were incubated in this solution 12-24 hours 4° C. The slides were rinsed in PBS for 1 hour, then incubated for 1 hour in the dark (at room temperature) with a 1:50 dilution of HRP-linked or FITC-linked goat anti-rabbit in PBS. After secondary antibody incubation, the slides were rinsed again for 1 hour in PBS. HRP-incubated slides were then reacted with metal-enhanced diaminobenzidine in stable peroxide buffer (Pierce.)

Since this procedure resulted in minimal immunostaining, some details were later changed. No blocking with goat serum was performed, and the primary antibody was diluted 1:50 with 10% normal goat serum (instead of BSA.) The slides were rinsed in PBS for 6 hours at 4°, then incubated simultaneously with 1:50 dilutions (in PBS containing 6% normal goat serum) of HRP-linked and FITC-linked goat anti-rabbit for 12-24 hours at 4°. The slides were then placed in PBS for at least 24 hours at 4° before viewing. Slides were first viewed with epifluorescence (excitation at 490 nm, emission at 520nm) on a Zeiss Research Microscope. The fluorescence was photographed with 90-second exposures on Kodak Ektachrome color slide film, ASA 200. The same slides, which had also been incubated with an HRP-linked secondary antibody, were then incubated with metal-enhanced diaminobenzidine substrate in stable peroxide buffer for at

least 1 hour before rinsing in PBS. Slides were air-dried overnight and sealed by coating with floor wax (Brite™, Johnson & Johnson.)

In later replications (indicated in figure captions), the primary antibody was diluted in PBS containing 1% normal goat serum, and the secondary antibody was diluted in PBS with no goat serum. No sodium meta-periodate etching was performed, and the slides were incubated with DAB 12-24 hours. In some cases, overstaining with DAB was corrected by dipping the slides in a 10% solution of commercially available bleach (resulting in a 0.6% hypochlorate solution) for 30-60 seconds, or until the color appeared suitable. The slides were air-dried and either coated with floor wax or sealed with a cover slip edged by nail polish.

Methoprene treatment of embryos

Horseshoe crab eggs were collected from adult females by manual stimulation of the gonadal pores. Eggs were placed in petri dishes in 0.2µm-filtered seawater. Sperm was collected from adult males, also by manual stimulation, and was applied to the eggs using a transfer pipette. Fertilized eggs were kept in aerated seawater at room temperature, with periodic addition of tap water to compensate for evaporation. After development of embryos occurred, twenty viable eggs were transferred to two new dishes of filtered (non-aerated) seawater (ten eggs in each.) Methoprene acid (purchased from Sigma) was diluted with ethanol to a concentration of 5mg/mL and was stored at -80°. This ethanolic stock solution was further diluted in filtered seawater and was applied to one dish of eggs at a concentration of 10 parts per million (ppm.), and then

100ppm. The second dish of eggs was treated with the same volume of ethanol alone as a control. The dishes were covered and kept at room temperature.

RESULTS

Capillary Electrophoresis

A. *Detection of lipid standards*

JH standard (dissolved in isopropanol) reliably produced a concentration-dependent peak when analyzed with CE (Fig 15.) The peak's retention time was usually around 40 min. A negative control (isopropanol) failed to produce this peak.

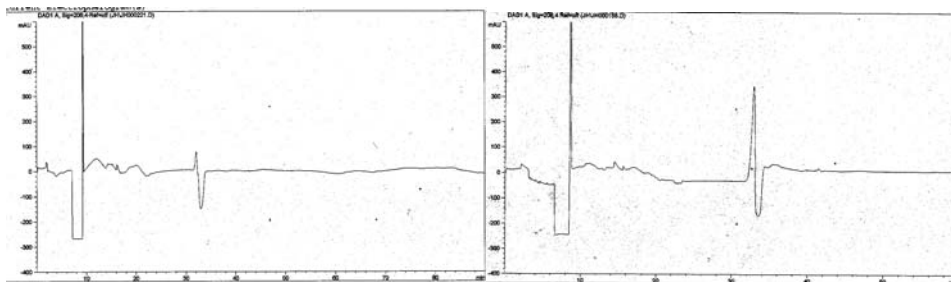


Fig. 15. CE electropherograms of JH III standard at two different concentrations. Right 1 μ g/mL; left: 0.1 μ g/mL.

When JH standard was subjected to the same treatment as the hemolymph samples (acetonitrile followed by hexane extraction) the peak was smaller and less reliably correlated with concentration; nonetheless, JH was still clearly detectable at around 40 min retention time (Fig. 16)

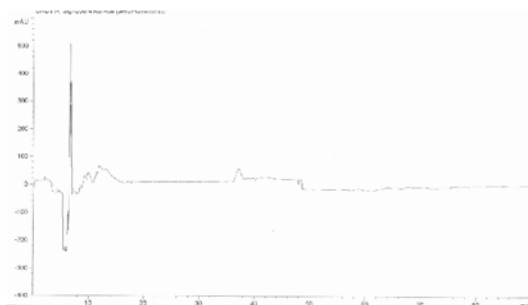


Fig. 16. CE chromatogram of JH III standard (1 µg/mL) after hexane extraction.

B. Test for antibody efficacy

In order to determine the efficacy of the antibody, CE analysis was performed on samples of JH standard that had been incubated with anti-JH and then spun and hexane-extraction to remove precipitated proteins. This procedure eliminated the presence of the previously observed peak (Fig 17.)

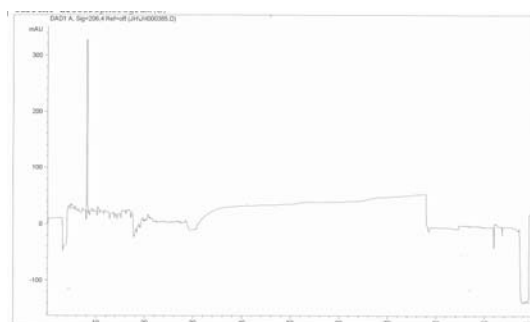


Fig. 17. CE electropherogram of JH III standard (300 µg/mL) after incubation with anti-JH (note the absence of the previously observed peak.)

C. Analysis of subadult hemolymph

C1. Hemolymph samples from a subadult male horseshoe crab, when analyzed via CE, produced a peak in the range of 30-50 minutes retention time, generally consistent with the retention time of the JH standard (Fig 18). Area under the peak appeared to be concentration-dependent.

C2. Subadult male hemolymph that had been incubated with anti-JH, as previously described, did not produce a peak (Fig 18).

C3. Subadult male hemolymph samples spiked with added JH consistently produced a single, very large peak at about 50-70 minutes retention time (Fig. 19).

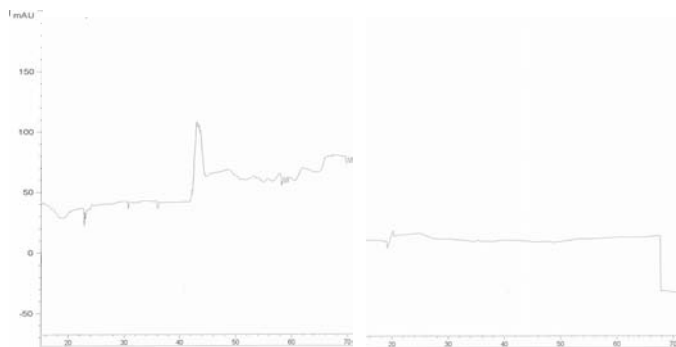


Fig. 18. CE electropherograms of juvenile male hemolymph. Concentration of the sample on the left is 40 times that of the sample in the middle (20 μ L vs. 0.5 μ L blood per 1mL prepared sample volume.) The rightmost sample was incubated with anti-JH prior to analyses; note the absence of the previously observed peak.

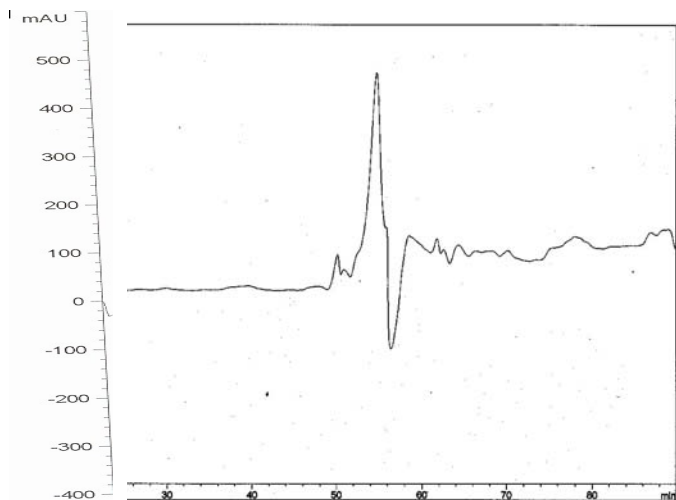


Fig. 19. Electropherogram of juvenile male hemolymph (0.5 μ L blood per 1mL sample volume) plus added JH III standard.

D. Analysis of adult hemolymph

Hemolymph samples from both male and female adult horseshoe crabs frequently failed to produce a discernible peak, indicating a possible lack of JH. In many analyses, a very small peak was present at 40 min retention time, possibly indicating low levels of circulating JH (Figs 20-21.).

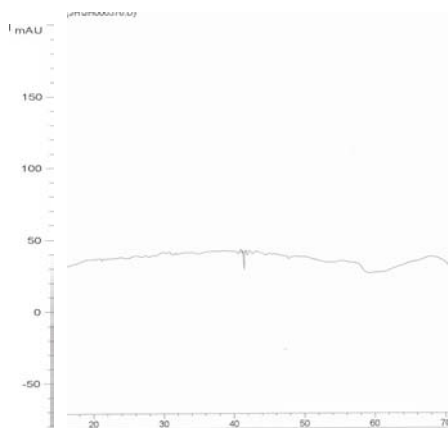


Fig. 20. Electropherogram of adult female hemolymph (20 μ L blood per 1mL sample volume.)

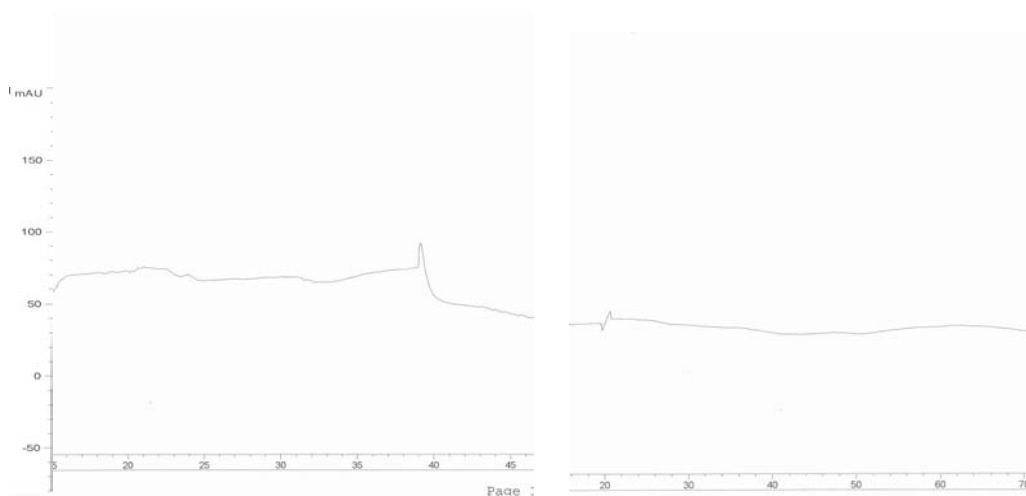


Fig. 21. Electropherograms of adult male hemolymph. Concentration of the sample on the left (10 μ L blood per 1mL sample volume) is five times that of the sample on the right.

E. *Analysis of insect hemolymph*

Hemolymph samples from tobacco hornworm caterpillars failed to produce a peak indicative of the presence of JH. Note that while the pooling of blood from

caterpillars of many sizes (see Table 1) was performed for HPLC analysis, the blood used for CE analysis was obtained from one or two animals only.

HPLC

Overall findings

HPLC analyses revealed a characteristic profile of peaks for all hemolymph samples analyzed, with a large peak (8-9 min RT), smaller peak (11-12 min RT) and in some cases smaller and broader peak(s) with RT = 20-25 min. Peak areas were significantly greater for the subadult horseshoe crab hemolymph and the insect hemolymph than for the adult horseshoe crab hemolymph. See Table 2 for a quantification of the levels of unoxidized JH in different blood samples, as calculated by the JH standard curve (Fig 22). Figure 23 represents a comparison of peak areas for all observed peaks.

<u>Animal</u>	<u>µg JH/mL</u>
Juv. female	92.1
adult male	66.5
adult female	45.5
new juv. male	91
stored juv. male	42

Table 2. Quantification of unoxidized JH levels in different animals, calculated from the JH standard curve (Fig. 22.)

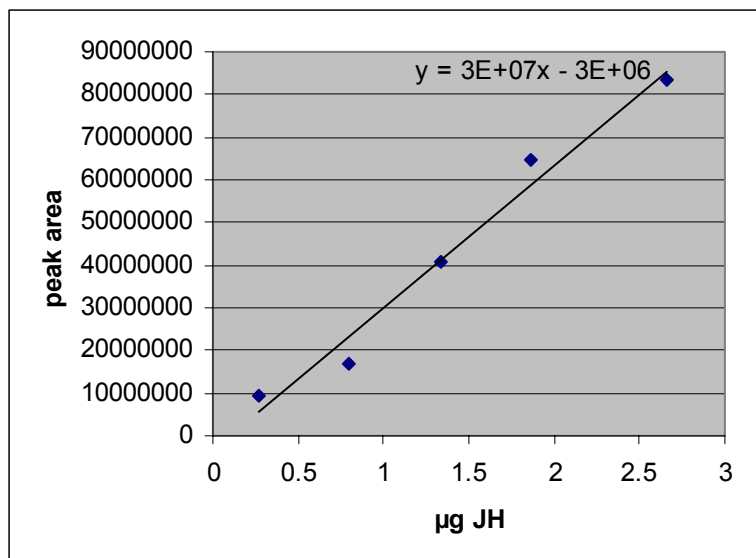


Fig 22. JH III standard curve.

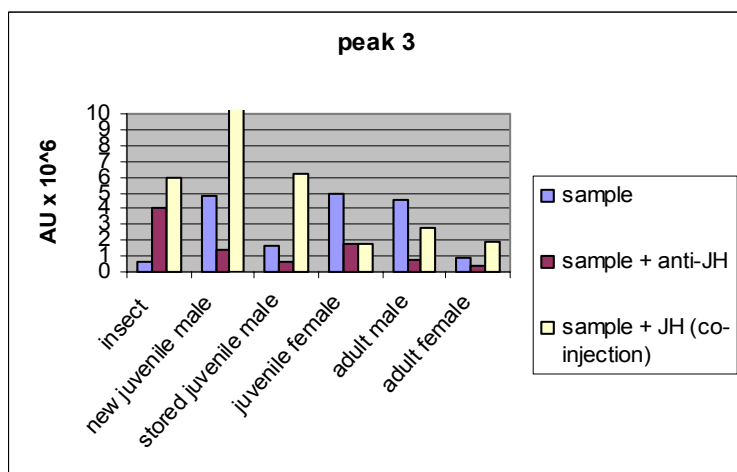
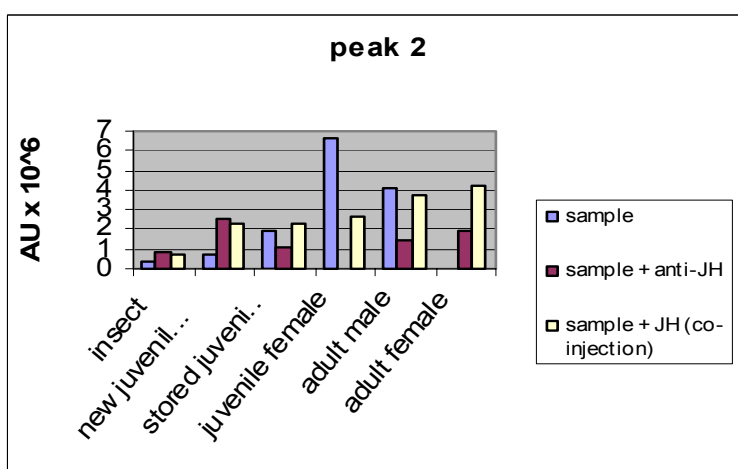
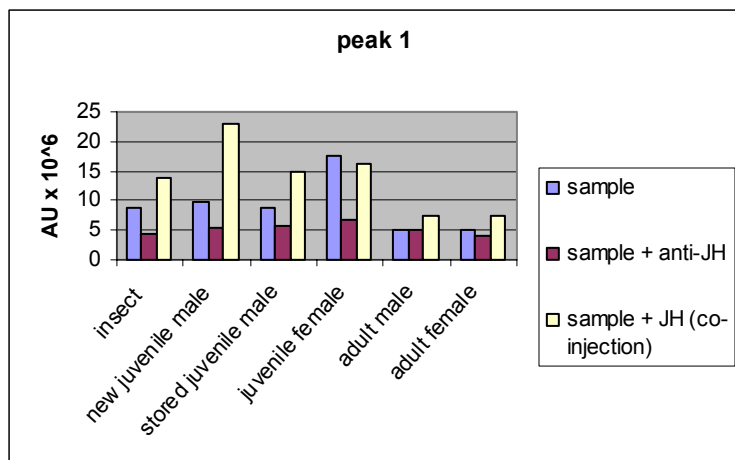


Fig. 23. Comparison of peak areas (in millions of absorbance units) of different hemolymph samples analyzed. Peak 1 represents the earliest peak that was consistently observed, with a retention time of 8-9 min, typically. Peak 2 represents the next peak, with a retention time of 11-12 min. Peak 3 had a retention time of 24-25 min.

A. Analysis of JH III standard

HPLC analysis of JH III standard resulted in a concentration-dependent peak with a retention time of around 20-25 minutes (Fig 24) The area of the peak was used to produce a standard curve (Fig 22) capable of quantifying amounts of JH or JH-like compound detected in hemolymph samples.

Later analyses of the same standard resulted in much lower sensitivity, a greatly reduced retention time (~10 minutes), and the appearance of several peaks (Fig 25.) These effects were likely due to storage of the hormone in a protic solvent (acetonitrile), as degradation of JH into several products is known to occur when such solvents are used for prolonged storage (Goodman, personal communication.) Despite the apparent degradation of the standard, peak areas were still concentration-dependent. New standard that was stored in hexane resulted in initial restoration of the previously observed 20-25 min retention time, but in approximately one week, the low sensitivity and shortened retention time characteristic of the degraded standard were again observed. According to associates at Sigma, this was likely due to exposure of the compound to air with resultant oxidation.

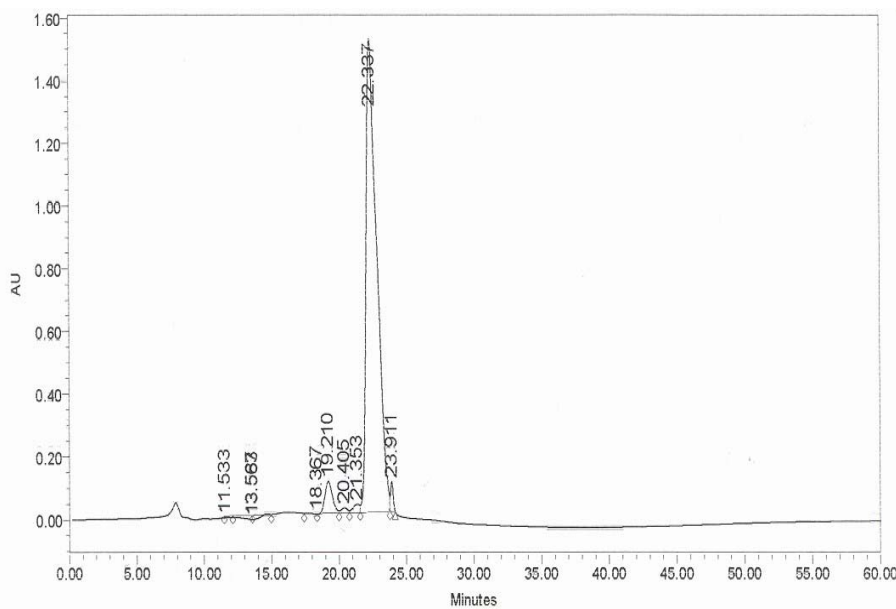


Fig. 24. HPLC chromatogram of new (unoxidized) JH III standard. Retention times of peaks are noted above each peak. Injection volume was 100 μ L; concentration was 100 μ molar.

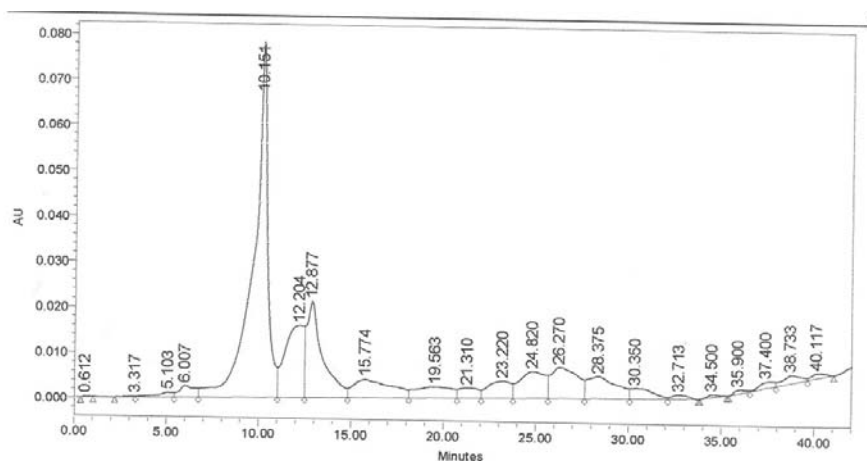


Fig. 25. . HPLC chromatogram of stored (oxidized) JH III standard. Retention times of peaks are noted above each peak. Injection volume was 100 μ L; concentration was 100 μ molar.

B. Analysis of subadult male hemolymph

Two samples of subadult male hemolymph (Fig 26 B and C) were analyzed: one that had been stored for several months at -20⁰C prior to hexane-extraction and evaporation (as described in the methods section), and one that was extracted and evaporated immediately after harvest.

Both samples showed a large peak with a retention time (RT) around 8-9 minutes, and a smaller peak with a RT of 11-12 minutes. The fresh (never frozen) samples also had two additional smaller peaks with RT equal to 19-20 and 24-25 minutes (Fig 26C.)

C. *Analysis of subadult female hemolymph*

Samples of subadult female hemolymph were extracted, evaporated, and analyzed without having been frozen or stored. Analysis showed three peaks (Fig 26E) roughly matching the subadult male hemolymph in profile: a large peak with RT = 8-9 min, a slightly smaller peak with RT = 11-12 min, and a small peak with RT = 25 min (Fig 26D.) Peak areas were larger for these samples than for the other hemolymph samples analyzed.

D. *Analysis of adult hemolymph*

All adult hemolymph had been stored at -20⁰C for several months prior to extraction. In both male and female hemolymph, peak profile corresponded to that found in the subadult hemolymph (peaks at 8-9, 11-12, and sometimes 24-25 min), but peak areas were generally lower (Fig 26E and F.)

E. *Insect hemolymph*

Hemolymph from *Manduca sexta* caterpillars produced a peak at 8-9 minutes, similar in size to those observed in the juvenile horseshoe crab hemolymph (Fig 26A). No significant additional peaks were observed.

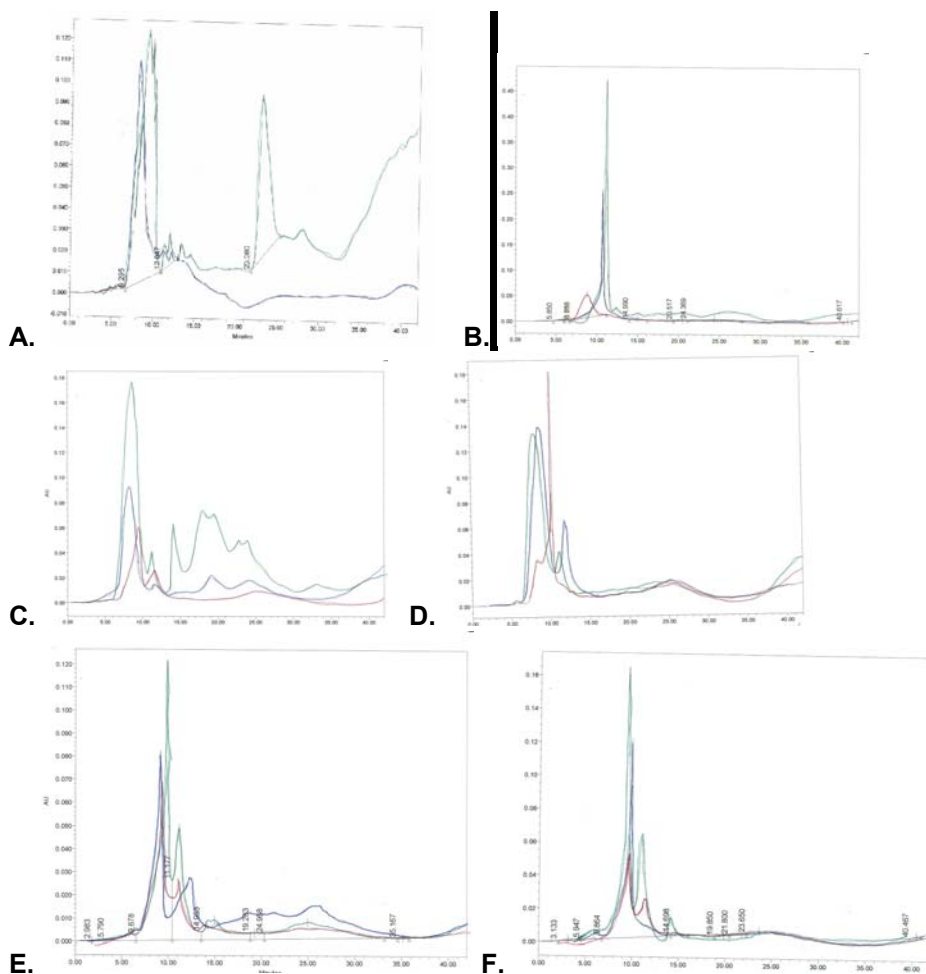


Fig 26. Overlaid chromatograms showing the blood sample alone (blue); the blood after incubation with anti-JH (red), and the blood co-injected with JH III standard (green.) A, *Manduca sexta* caterpillars; B, juvenile male *Limulus* (after frozen storage for several months); C, juvenile male *Limulus* (never stored); D, juvenile female *Limulus*, E, adult male *Limulus*, F, adult female *Limulus*. Note that antibody data have been omitted from (A) since those data were inconclusive.

F. Response to anti-JH

In virtually all cases (see figs 26A-F, and Fig 23), hemolymph that had been incubated with anti-JH prior to extraction showed greatly reduced peak areas when analyzed. Significant size reduction of the earlier two peaks usually occurred, while the third peak (RT = 24-25 min), when present, was obliterated entirely.

G. Co-injection of JH standard with hemolymph samples

In most case (see Figs 26A-F, and Fig 23), the area of the earliest peak observed in the hemolymph analyses was significantly increased by the co-injection of JH standard. The third peak, when present, was usually also increased.

H. Analysis of blood cell lysate

Lysate from both juvenile male and juvenile female blood cells (Fig 27 A and B) produced profiles very similar to those for the blood serum, with larger peaks of RT = 9-10 and 11-12 min, and a smaller peak with RT = 24-25 min.

The later peak was significantly larger in the female.

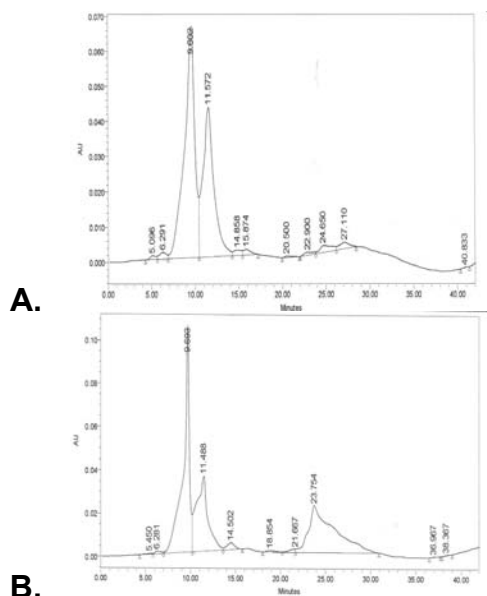


Fig. 27. HPLC chromatogram of blood cell lysate from subadult male (A) and subadult female (B) blood. Analysis volume was approximately equivalent to 300,000 amoebocytes, assuming a concentration of 5000 amoebocytes/ μ L in whole blood.

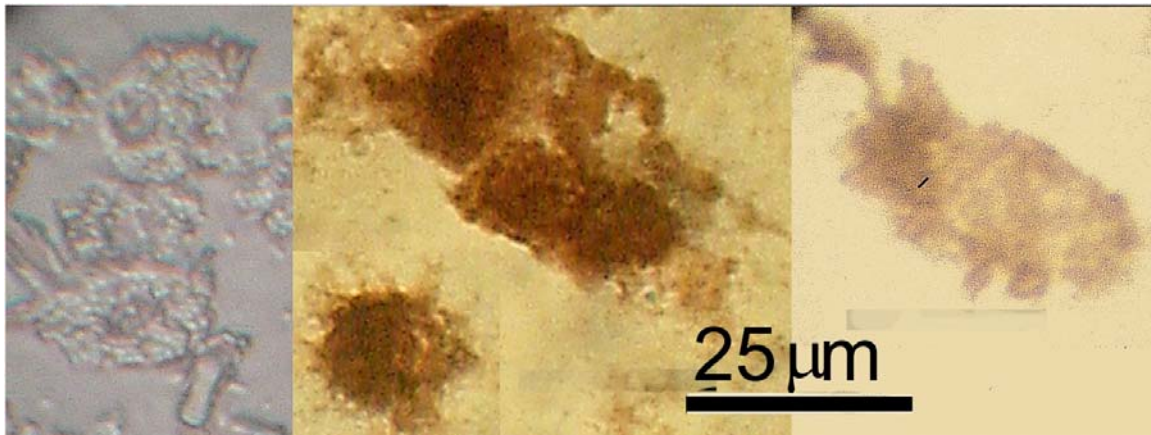
IMMUNOHISTOCHEMISTRY

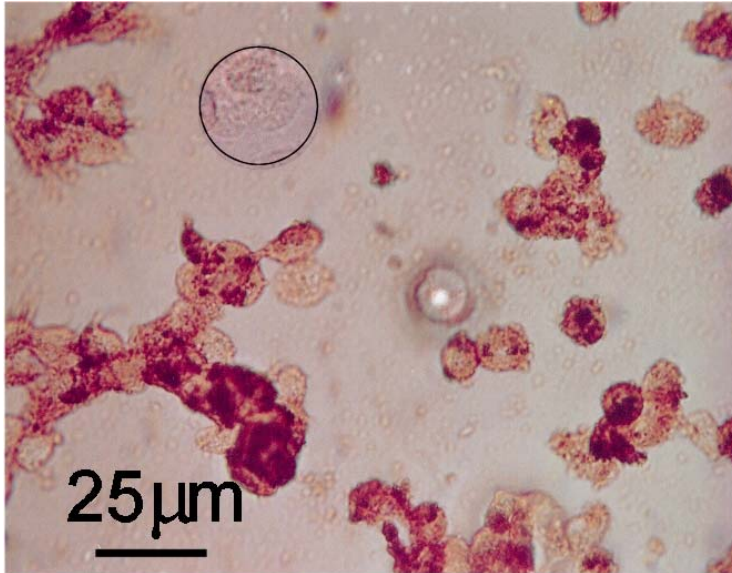
Wholemounds

FITC immunostaining of whole nerve cords of horseshoe crabs and *Manduca sexta* caterpillars yielded ambiguous results. Some fluorescence (other than autofluorescence of chitinous tissues) was observed, but it was impossible to determine whether the observed fluorescence indicated the presence of JH, or the organ or cells in which it was concentrated, either for release or as a result of uptake.

Amoebocytes incubated with anti-JH primary and HRP-linked secondary antibodies resulted in dark staining, indicating high levels of JH (Fig. 28). FITC-linked secondary antibody produced observable fluorescence in amoebocytes (Fig. 29.) Homogenized eggs failed to produce any positive results from immunostaining either with HRP- or FITC-linked secondary antibody.

A.

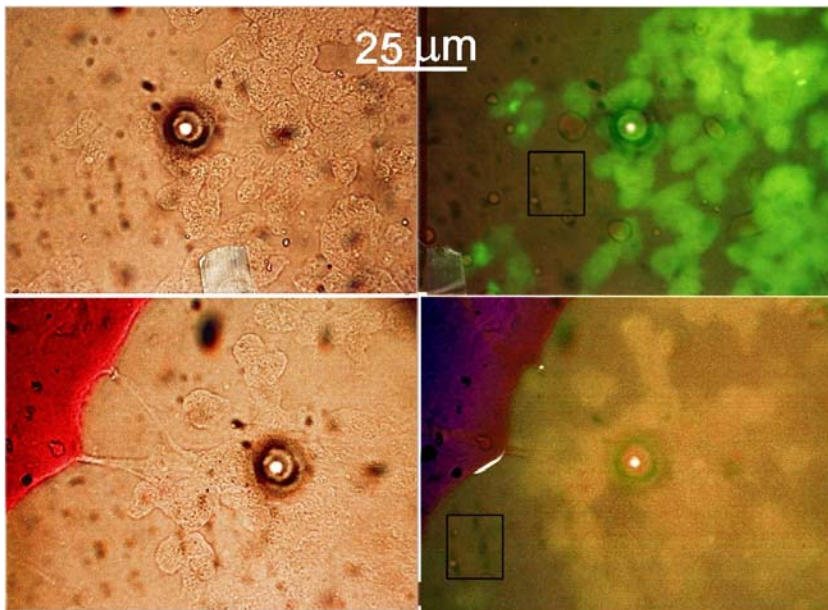




B.

Fig 28. **A.** Immunostaining of amoebocytes (from a subadult) with anti-JH primary and HRP-linked secondary antibodies. Left, live cells. Center, experimental slide incubated with both antibodies. Right, control slide incubated with secondary antibody only. **B.** Immunostaining of amoebocytes (from a 42mm-long juvenile) with anti-JH primary and HRP-linked secondary antibodies. Inset circle: control cells incubated with secondary antibody only.

A.



B.

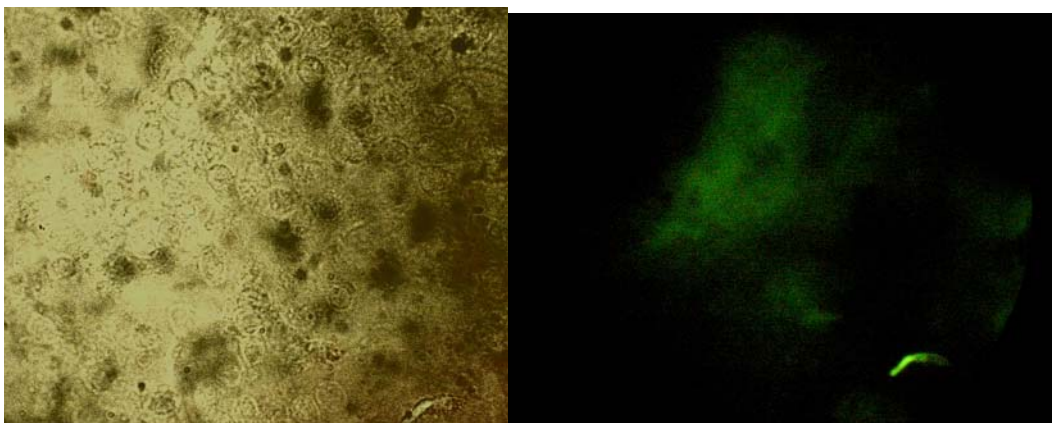


Fig. 29. **A.** Immunostaining of amoebocytes (from a 42-mm juvenile), incubated with anti-JH as primary and FITC-linked GAR as secondary. Top row, experimental slide with both antibodies; bottom row, control with primary antibody only. Viewed with transmitted light, left; viewed with epifluorescence, right. **B.** Immunostaining of amoebocytes (from a subadult), incubated with anti-JH as primary and FITC-linked GAR as secondary; viewed with transmitted light, left; viewed with epifluorescence, right. Control had no fluorescence and is not shown.

Frozen sections

Because of the large amount of soft tissues in the juvenile animals used for this procedure, frozen sectioning was largely unsuccessful. The sections consistently fell apart during or after sectioning, and so we decided not to proceed with immunostaining and microscopy of these specimens.

Embedded sections

Initial fixation and embedding of whole larvae and first-stage juveniles resulted in soft interior tissue that dissolved in the electron beam (an indication of incomplete infiltration with resin, often a problem with yolk material.) Later attempts resolved this problem, but upon viewing, grids were seen to be contaminated with large electron-dense particles (Fig 30). These particles were found in the secondary antibody, and they made it impossible to determine

immunogold reactivity. Single colloidal gold particles were also observed, but they were not localized to any specific cells or tissues. Even though new colloidal gold goat anti-rabbit antibody was purchased, we decided not to re-do this procedure because no immunoreactivity was found in the nervous system on thick sections of the same tissue examined by light microscopy.

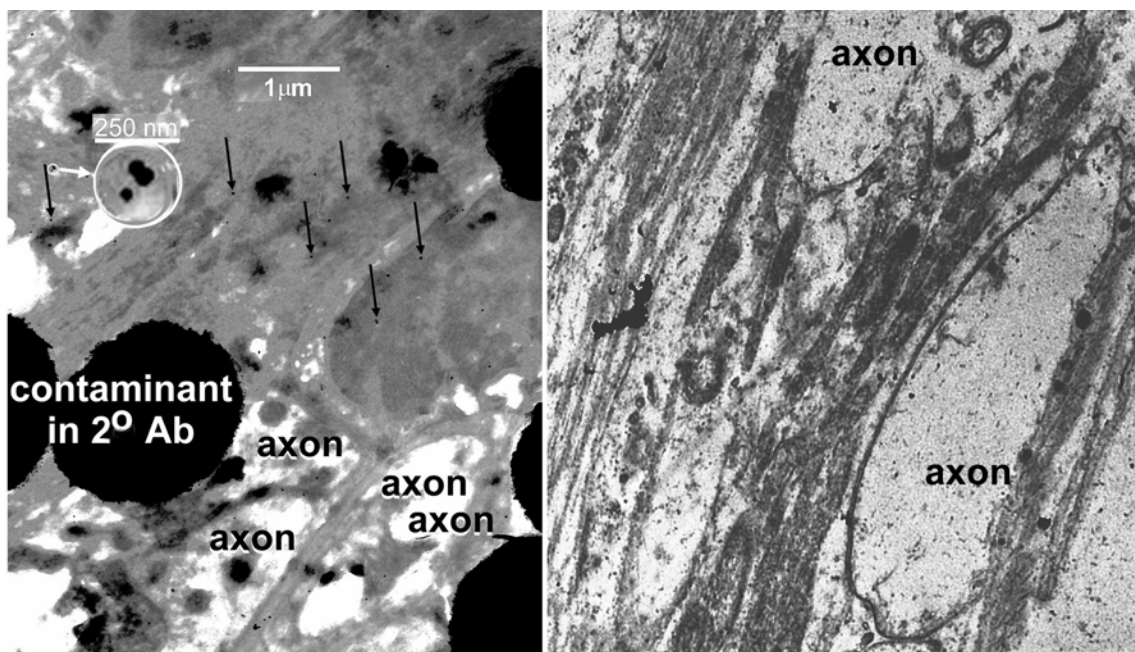


Fig. 30. TEM micrographs of *Limulus* neural tissue showing the presence of contaminating particles in the secondary antibody. Left, sample incubated with anti-JH primary and immunogold-linked secondary (both 10nm and 25nm gold particles; see arrows.) Right, sample incubated with primary antibody only.

Immunostaining for light microscopy, done on larvae that had been sectioned laterally, showed immunoreactivity with both FITC-linked (Fig 31) and HRP-linked (Fig 32) secondary antibodies. Immunoreactivity was concentrated in lipid droplets originating from the yolk cells, present in the digestive system of the larvae. Although some background staining was present in control sections that were not incubated with the primary antibody (HRP, Fig 32, right; and

fluorescence, Fig 31, right), staining is much lighter than in experimental sections. No reactivity was observed in the nervous system.

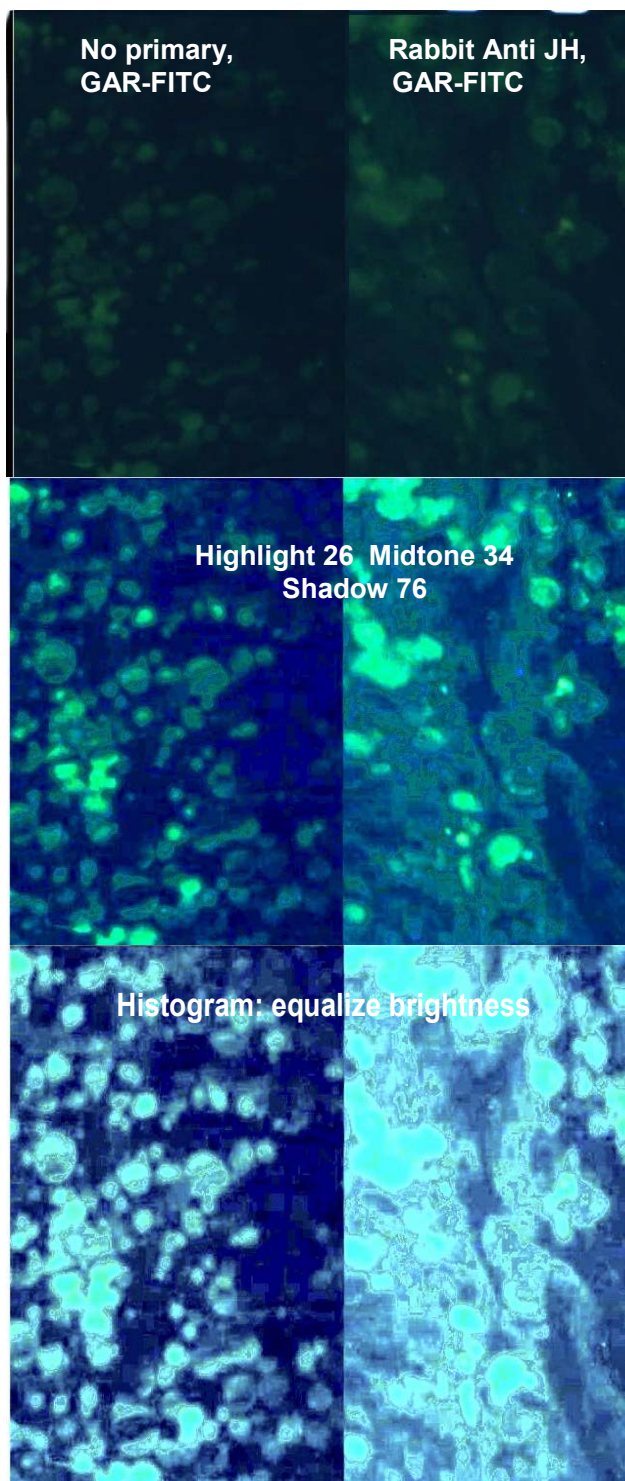


Fig. 31. FITC reactivity in the digestive tract of a larval horseshoe crab. Round particles are lipid droplets from ingested egg yolk. Left halves of pictures are those with no primary antibody; right halves have anti-JH as primary antibody. Image modifications, performed in Microsoft Photo Editor, are noted.

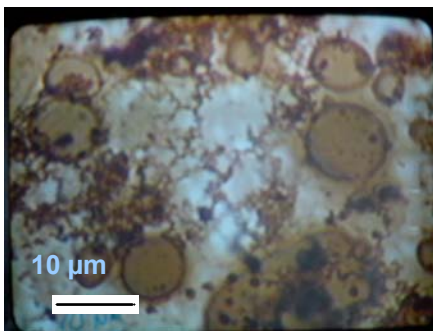
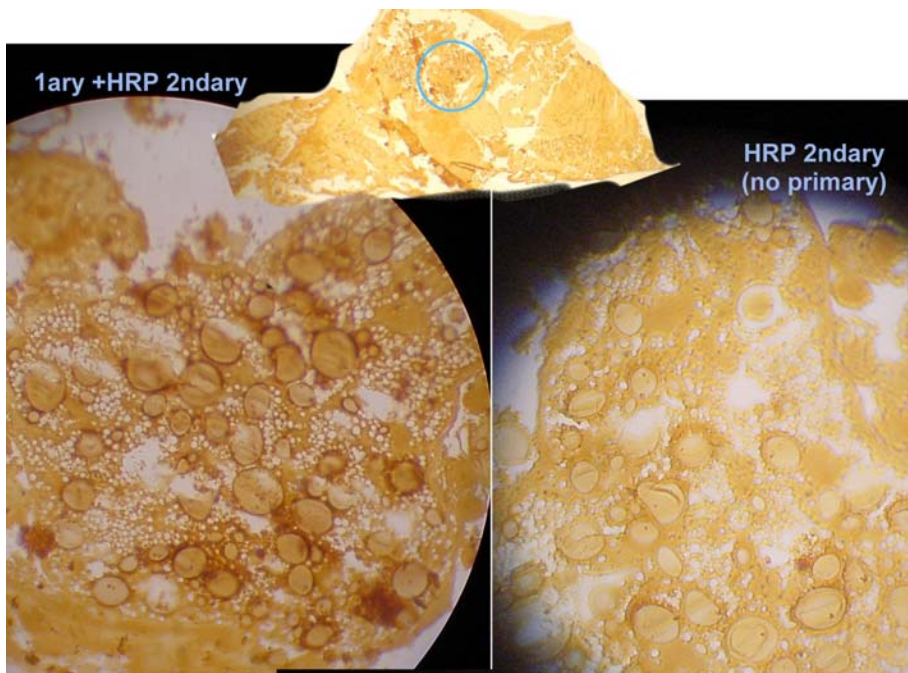


Fig. 32. HRP reactivity (using anti-JH as primary antibody) in the digestive tract of a larval horseshoe crab. Anti-JH was used as primary antibody in the specimen on the left; no primary antibody was used on the right. Inset shows the entire cross-sectional slice of the animal with the enlarged area circled. Note the immunostaining of the perimeter of lipid droplets originating from the egg yolk. Another view of the experimental slide is included below for scale (each lipid droplet is approximately 5-10 μ m in diameter.)

Further replications of incubation with HRP-conjugated secondary antibody confirmed this finding. Experimental sections consistently showed dark staining on the perimeter of lipid droplets, while control sections had a much more uniform background stain. Apparent adherence of the secondary antibody to the glass of the slide (as evident by the “flocked” brown appearance of non-tissue slide areas) was much more noticeable in the experimental slides than in the controls. This is likely due to the fact that these slides were incubated in a greater depth of solutions than were the controls, resulting in settling of precipitates on the surface of the glass.

JH Analog and Anti-JH Treatment of Live Specimens

Initial treatment of eggs containing late-stage embryos (N=10) with 10ppm methoprene yielded no observable difference from the ethanol control. Hatching occurred in both groups. When the concentration of methoprene was increased to 100ppm (with a corresponding increase in the volume of ethanol), most of the animals died. Those that lived (2 experimental and 1 control) were paralyzed and could move only slightly when prodded.

DISCUSSION

Capillary Electrophoresis

Development of a successful CE detection method for this compound proved problematic. Capillary clogging, erratic current, and varying retention times

occurred frequently. Some changes to the procedure, as noted here, helped alleviate some of the problems.

Finding a satisfactory solvent for the samples was one difficulty. Using the run buffer as a solvent would have been optimal, since it would introduce no new substances into the analysis, but in this case the hydrophobic samples were insoluble in the run buffer. Thus, various organic solvents were tried instead. Since de Oliveria's (2001) fatty acid analyses used methanol as a solvent, this was tried first, but capillary clogging occurred frequently. This outcome is consistent with other studies (e.g., Lee et al 1999, Magnusdottir et al 1999), which have found that frequent capillary clogging can occur due to the "stacking effect," which is particularly likely to occur with lipophilic analytes in organic solvents. Stacking effect refers to a buildup of analyte that can occur when the sample is dissolved in a solvent with a lower ionic strength than the run buffer. Varying the proportions of buffer to solvent can mediate the stacking effect, with a higher buffer percentage promoting less stacking and therefore less propensity to clog. In this case, both methanol and ethanol proved unusable since there was no effective buffer/alcohol ratio found—high proportions of alcohol caused clogging while high proportions of buffer rendered the samples insoluble. Isopropanol seemed more effective, and through a series of attempts, a 30% isopropanol/70% buffer solution was found most effective.

Capillary clogging still occurred fairly frequently even with this solvent. This seemed to be mitigated somewhat by more stringent between-run flushing procedures. The most effective flush procedure was that described in the

Methods section (15 min each of 1N NaOH, 0.1N NaOH, water, plus 25 min buffer.) Acid-base flushing (1M HCl followed by 1M NaOH), nonionic detergent (Triton-X), and shorter flush times all led to greater frequency of clogging.

Besides the stacking effect and residual adherence of analyte to the capillary walls, the formation of precipitates in sample vials was another possible source of capillary clogging. The potential for this problem was eliminated by using each vial of sample or buffer for one run only. After a single analysis, vials were either discarded or the contents were refiltered into a new vial.

Other CE analyses of fatty acids have also reported frequent clogging, poor reproducibility, and unstable current. Some researchers attribute this to adsorption of the surfactant to the capillary surface (Collett & Gareil 1997). Others have alleviated clogging by trying alternative buffer additives, such as urea, to improve analyte solubilization (Emara et al 2001).

Detection at both 206nm (maximal fatty acid absorption) and at 224nm (maximal absorption of SDBS, the surfactant present in the buffer) was used, with little difference observed in the electropherograms.

Presence of a single peak in analyzed blood samples is troubling, since whole blood probably contains multiple lipid-based components that should be separated by this CE method. The single peak could indicate that a single lipid component (possibly JH) is present at much higher concentrations than other such molecules. It could also indicate that this method fails to reliably separate and/or resolve different lipids. Analyses of blood spiked with JH also produced a single peak, which may either mean that this peak is also JH (or is similar

enough to co-migrate, possibly through dimerization), or again, that the method is inadequate for resolution of different lipids. Although I attempted to answer this question by spiking blood with a different lipid-based hormone (ecdysone), results from these tests were inconclusive, due to recurrent problems with current instability and clogging.

Detection of a JH III standard using this CE method was successful, but further refinement of the method is still needed to improve replicability. Problems that need to be addressed include capillary cleaning/flushing procedures, and possibly alteration of the buffer composition to include a different surfactant, or a different concentration of surfactant, as well as possible other components such as flow modifiers. Widely varying retention times, background “noise,” and persistent problems with capillary clogging and current instability indicate a probable failure of between-run flush procedures to remove all traces of analyte molecules from the capillary. The very long retention times (and possibly, again, the variation in these times) may well be related to the type and concentration of surfactant in the buffer. Changes to this parameter may improve performance.

Despite these consistent problems with CE analyses, some data were obtained. Detection of JH standard was successful, with a peak appearing at around 40 min retention time. Hemolymph from juvenile male horseshoe crabs also consistently produced a peak with a similar RT; this peak was significantly reduced in antibody-incubated samples. Adult (male and female) hemolymph samples frequently produced no peaks, but at high concentrations, small peaks with ~40 minute RTs were sometimes observed. Although the problems

encountered with this method of analysis make it difficult to draw definitive conclusions, these observations suggest JH present in high levels in juveniles and low levels in adults.

HPLC

Because of the aforementioned problems encountered with some CE analyses, HPLC was used to confirm the conclusions obtained from CE. HPLC analyses of new JH III standard yielded a peak with a retention time of 20 – 25 minutes. Many analyses of the horseshoe crab hemolymph also produced a peak with a similar retention time; the obliteration of this peak with the addition of anti-JH is strong evidence that this peak represents JH.

In addition to this 20-minute peak, hemolymph analyses also produced two (sometimes more) earlier peaks with retention times typically 8-9 and 11-12 minutes. JH standard that had been stored for longer than a few days, resulting in oxidation, also produced peaks with these retention times. JH III contains multiple double bonds, and its potential for oxidation was illustrated to us when a degraded sample of juvenile hormone was shipped to us by Sigma Chemical Company. Sigma's only recommendation for storage is to keep it at -20 C, and they do not ship JH III on dry ice, nor do they add an antioxidant. It arrives packed in the inert gas argon, but the lability of JH III is not adequately discussed in the packing instructions or on the vial. Oxidation products of JH III would be more polar and have larger molecular weights than the original compound, resulting in less column affinity; this is consistent with the observed earlier

retention time. These early peaks noted in hemolymph analyses were consistently reduced in size after sample incubation with anti-JH, and were increased in size with the co-injection of JH standard. These findings strongly suggest that these peaks represent a similar compound to the oxidized JH standard. JH is a short-lived compound in physiological systems, and is highly labile under storage conditions. The presence of this oxidized JH-like compound in hemolymph samples may therefore either indicate the presence of JH metabolic breakdown products in the blood prior to harvest, or post-harvest oxidation of native JH found in the blood. Because of the labile nature of JH, it is likely that the antiserum used in these experiments may actually contain a mixture of antibodies against both native JH and its oxidation products. If the JH used for inoculation in creating this antiserum were, in fact, already oxidized or partially oxidized, then the antibodies thus produced would be effective against the oxidized as well as the native forms of the hormone.

The 25-minute peak, representing native JH, was most prominent in the sample of juvenile male hemolymph that had never been frozen or stored. In the sample of frozen juvenile male hemolymph, a peak with this RT is barely discernible. Such a comparison suggests that the oxidation of JH is occurring post-harvest. However, lack of this 25-minute peak in the pooled blood from hornworm caterpillars may suggest a physiological breakdown of the hormone. Since intermolt periods are far shorter in insects than in horseshoe crabs (a period of hours rather than months or years), JH breakdown would occur very rapidly in insects; if JH levels remained high, ecdysis would not occur. For this

reason, it would be difficult to find native JH in insect blood, even when samples from many animals were pooled (but the oxidation products occurring as a result of breakdown would still be present.) Future studies in this area could resolve the question of pre- or post-harvest oxidation by immediately adding an antioxidant to drawn blood. In addition, analysis of this oxidation product via gas chromatography/mass spectroscopy would be useful to identify its molecular structure.

A well-known parallel to the observed easily-oxidizable nature of JH can be seen in the prostaglandins. Prostaglandins, like the juvenile hormones, are fatty-acid-derived, hormone-like molecules that exert a range of functions on many physiological systems. As lipid derivatives, they convert easily into oxidized byproducts, failing to survive even a single pass through the lungs (Vovalles et al 1973.) It is likely that a similar oxidation occurs in arthropods with respect to JH.

Areas of all peaks (both native and oxidized JH) were significantly larger in juvenile animals than in adults, a finding consistent with the known functions of the juvenile hormones. Contrary to our original hypothesis, adult females do not appear to retain high levels of circulating JH. Such a finding suggests that despite the lack of an easily observed change in morphology, female horseshoe crabs probably do undergo a terminal molt upon reaching maturity, at which time circulating JH levels drop.

HPLC analyses of blood cell lysate indicated that JH (both oxidized and unoxidized) is present in cells as well as in serum. *Limulus* blood carries oxygen

on hemocyanin, a copper-containing protein free in the serum. Oxygenated hemocyanin might oxidize serum JH, so the portion of JH carried inside the amoebocytes, the only blood-borne cell type, might be better protected against oxidation. Amoebocytes are present at about the same concentration as human white blood cells (5000 per microliter: Flynn 1997) and their documented function is crudely immunological: they produce a clotting cascade in the presence of gram-negative bacteria. JH seems to enter these cells easily—a predictable effect of its lipophilic nature. Strong indications of cellular JH were also obtained from immunohistochemical experiments (see below.)

Immunohistochemistry

Localization of this lipid-based compound proved to be a particularly problematic application of immunohistochemistry techniques. Osmium postfixation was used to eliminate the possibility of extracting the hormone during ethanol dehydration steps; however, osmification contributes its own problems since the resultant cross-linking of unsaturated bonds may affect the antigenicity of the compound. TEM immunostaining procedures used here also involved detergent (Triton-X or Tween-80) rinses, a typical preparation step for resin-embedded specimens that would not affect proteins but may have extracted lipid-based molecules or destroyed their antigenicity.

Light microscopy proved to be a more reliable immunolocalization technique than electron microscopy. Two distinct immunolocalization techniques (FITC and HRP) support the presence of a JH-like antigen in, or surrounding,

lipid droplets located in the larval digestive tract. These droplets are remnants of egg yolk, upon which the larvae continue to feed until they begin taking in food from external sources. Maternal deposition of hormone in the eggs prior to laying is most likely the source of JH in these droplets. It is not only possible but likely that endogenous production of the hormone may begin after the larvae use up the yolk stores and are no longer receiving maternal hormone. This would account for Jegla and Costlow's (1982) observation that exogenous JH applications did not affect embryonic or larval development. Their conclusion was that JH was not involved in *Limulus* development, but ours is that endogenous JH is abundant and makes any additional source of JH superfluous.

JH's lipophilic nature makes it likely that this hormone permeates membranes and so should be found in blood cells and in eggs. The presence of JH in blood cells was suggested, as noted above, by HPLC. We also performed immunohistochemistry on collected blood cells, which yielded a dramatic response to anti-JH, indicating large amounts of hormone present. Despite the presence of JH in embryos, little or none was detected in the pelleted extracts of fertilized eggs. JH, however, either has some solubility in water-based fluids, or has a carrier protein that makes it soluble. It is therefore possible that the hormone was thrown out with the "bathwater," and the supernatant from egg homogenates should be tested as well.

JH Analog and Anti-JH Treatment of Live Specimens

Treatment of live larvae with the JH analog methoprene produced inconclusive findings, since the toxicity of the ethanol vehicle prevented observation of methoprene's effects. In future studies, this experiment should be modified and repeated to determine whether methoprene exerts any effect on *Limulus*. Because of the other results obtained here, as well as prior studies indicating ticks' susceptibility to JH analogs, it is highly likely that a developmentally disruptive effect would be observed.

Treatment of live larvae or juveniles with anti-JH is another future experiment that could provide useful supporting data for our conclusions. Inactivation of endogenous JH by injection of the antibody should result in premature appearance of adult characteristics (such as claspers in the male), despite a juvenile size.

CONCLUSIONS

These data present evidence that the horseshoe crab, *Limulus polyphemus*, does produce a lipid-based molecule structurally and functionally similar to insect JH. Presence of this compound at detectable levels in subadults but not adults further bolsters the evidences that this is a juvenile hormone, which likely has similar functions in *Limulus* to those of JH III in insects (i.e, maintaining the juvenile form by blocking the effects of ecdysone.) We initially hypothesized that adult female horseshoe crabs may have high circulating JH levels that are responsible for their (apparent) lifelong growth and lack of secondary sex characteristics. However, these data do not support that

conclusion: analysis of adult female hemolymph, as well as that of adult male hemolymph, indicated only low concentrations of JH.

Our data indicate that this JH-like compound is easily oxidized, and that both the oxidized and native forms retain the epitope necessary for antibody interaction. The oxidized form was present at much higher concentrations than the native form, particularly in stored samples, in which the native form was barely detectable. The oxidation may occur in the physiological system, or during storage and processing of the harvested blood.

Localization experiments revealed (contrary to our expectations) that the compound is not detectable in neural tissue. Detectable levels of this hormone were instead found in the larval digestive tract and in the blood cells.

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