2000

# Absence of Insect Juvenile Hormones in the American Dog Tick, Dermacentor veriabilis (Say) (Acari: Ixodidae), and in Ornithodoros parkeri Cooley (Acari: Argasidae)

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Prepared for:
Journal of Insect Physiology

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# Absence of insect juvenile hormones in the American dog tick, *Dermacentor variabilis* (Say)(Acari:Ixodidae), and in *Ornithodorus parkeri* Cooley (Acari:Argasidae)

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Received; ??/??/??; received in revised form ??/??/??; accepted ??/??/??

**Abstract**

Synganglia, salivary gland, midgut, ovary, fat body and muscle alone and in combination from the ixodid tick, *Dermacentor variabilis* (Say), or the argasid tick, *Ornithodoros parkeri* Cooley, were incubated *in vitro* in separate experiments with L-[*methyl*-$^{3}$H]methionine and farnesoic acid or with [1-$^{14}$C]acetate. Life stages examined in *D. variabilis* were 3-72 h old (after eclosion) unfed nymphs, partially fed nymphs (18-72 h after attachment to the host), fully engorged nymphs (2 d after detachment from host), 3-72 h old (after eclosion) unfed females, partially fed unmated females (12-168 h after attachment to host) and replete, mated females (2 d after detachment from the host). Those from *O. parkeri* were last stadium nymphs and female *O. parkeri*, all 1-2 h after detachment. Corpora allata from *Diploptera punctata*, *Periplaneta americana* and *Gromphadorina portentosa* were used as positive controls in these experiments. No farnesol, methyl farnesoate, JH I, JH II, JH III, or JHIII bisepoxide was detected by radio HPLC from any tick analysis while JH III, methyl farnesoate, and farnesol were detected in the positive controls. To examine further for the presence of a tick, insect-juvenilizing agent, *Galleria* pupal-cuticle bioassays were conducted on lipid extracts from 10 and 15 d old eggs, unfed larvae, unfed nymphs, and partially fed, unmated female *D. variabilis* adults. Whole body extracts of fourth stadium *D. punctata* and JH III standard were used as positive controls. No juvenilizing activity in any of the tick extracts could be detected. Electron impact, gas chromatography-mass spectrometry of hemolymph extracts from fed, virgin and replete female *D. variabilis* and fully engorged female *O. parkeri* also failed to identify any of the known insect juvenile hormones in these ticks. The same procedures were successful in the identification of JH III in hemolymph of fourth stadium *D. punctata*. Last stadium nymphal *O. parkeri* implanted with synganglia from next to last nymphal instars underwent normal ecdysis to the adult. The above studies *in toto* suggest that *D. variabilis* and *O. parkeri* do not have the ability to make the common insect juvenile hormones, and

juvenile hormone does not regulate tick metamorphosis or reproduction as hypothesized extensively in the literature.

*Keywords:* American dog tick; *Dermacentor variabilis*; *Ornithodoros parkeri*; Juvenile Hormone; Embryogenesis; Metamorphosis; Vitellogenesis

Footnotes

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#Abbreviations used: AMU, atomic mass unit; $CD_3OD$, methyl-$d_3$-alcohol-d; $CHCl_3$, chloroform; $d_3$-MH, trideuterated methoxyhydrin; EI, electron impact; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; JH, juvenile hormone; JH I, juvenile hormone I; JH II, juvenile hormone II; JH III, juvenile hormone III; $JHA_3$, juvenile hormone III acid; $JHB_3$, juvenile hormone III bisepoxide; $JHD_3$, juvenile hormone III diol; MF, methyl farnesoate; NaOH, sodium hydroxide; OTFP, 3-octylthio-1,1,1-trifluoropropan-2-one.

## 1. Introduction

Our understanding of the role of juvenile hormone (JH#) in the regulation of arthropod development is based primarily on research within the Mandibulata. JH, produced in insects by the corpora allata, regulates growth, metamorphosis, reproduction, behavior (Laufer and Borst, 1983) and diapause (Walker and Denlinger, 1980). JH so far has only been found in the Insecta. In Crustacea, the mandibular organs produce methyl farnesoate (MF, a methyl ester of farnesoic acid and a precursor to JH III). MF in crustaceans regulates the duration of the intermolt period (Yudin et al., 1980; Homola and Chang, 1997) and initiates vitellogenesis (Hinsch, 1980; Laufer et al., 1986; Sagi et al., 1993; Homola and Chang, 1997), but unlike insects, does not control metamorphosis (Homola and Chang, 1997).

The general assumption has been that the role of JH in the regulation of metamorphosis and reproduction in ticks is similar to insects (summarized by Obenchain and Galun, 1982; Sonenshine, 1991). The strongest evidence for JH in ticks was demonstrated by the studies of Pound and Oliver (1979). The anti-allatotropin, precocene II, disrupted egg development and oviposition in *Ornithodoros parkeri*, which could be partially rescued with low but not high doses of JH III. Leahy and Booth (1980) also demonstrated that precocene II sterilized ixodid and argasid ticks similar to that observed in the Heteroptera (Manser et al., 1979). Obenchain and Mango (1980) and Connat et al. (1983) reported JH-mediated gonadotropic effects after the topical application of JH I, JH III and JH mimics on fed, virgin *O. moubata*. However, Taylor et al. (1991) were unable to induce vitellogenesis with these same JHs or the JH mimic, methoprene, in unfed *O. parkeri* even though the pyrethroid insecticide, cypermethrin, was able to stimulate egg maturation in control experiments.

There is also equivocal pharmacological evidence for a role for JH in tick molting and metamorphosis. Leahy and Booth (1980) found that the topical application of precocene II after engorgement inhibited molting in larvae and nymphs of *Argas persicus* and *Rhiphicephalis sanguineus*, but

there was also a high level of mortality. Khalil et al. (1984) applied JH I topically to nymphs of the camel tick, *Hyalomma dromadarii*, and found delayed molting only when the treatment was made at the time of repletion. No evidence of additional juvenile stages due to a JH or juvenoid treatment has been reported in ticks (Sonenshine, 1991).

Venkatesh et al. (1990) and Roe et al. (1993) measured low levels of JH esterase and JH epoxide hydrolase activity during *D. variabilis* nymphal and adult development. Sonenshine et al. (1989) and Lomas et al. (1996) described proteins in *D. variabilis* and *Amblyomma herbraeum*, respectively, that bound JH. Roe et al. (1993) found that *D. variabilis* synganglia incubated *in vitro* with L-[*methyl*-$^{3}$H]methionine produced radiolabeled products in low amounts that were soluble in hexane and had similar mobility to know JH standards using normal phase thin layer chromatography. Connat (1987) discovered JH-positive immunoreactive material but was unable to definitively identify JH using HPLC-coupled radioimmunoassays in hemolymph from *Boophilus microplus*.

Essential to future progress in understanding the endocrine regulation of development, metamorphosis and reproduction in ticks is the determination of the presence or absence of JH. In this paper, we address this question by measuring JH biosynthesis in hard and soft ticks at different developmental stages and in different tissues and by the direct assay for JH or juvenizing compounds using the *Galleria* pupal-cuticle bioassay, transplantation experiments and electron impact, gas chromatography-mass spectrometry.

## 1. Materials and methods

### *2.1. Tick rearing*

A colony of *Dermacentor variabilis* (Say) reared from specimens originally collected near Richmond, VA was maintained during their non-parasitic phase in a controlled environmental chamber at 27± 0.5°C and 90± 2% relative humidity with a daily photophase of 14 h. *D. variabilis* larvae and nymphs were fed on Norway rats, *Rattus norvegicus*, while adults were reared on albino rabbits, *Oryctolagus cuniculus* (Sonenshine, 1993). *Ornithodoros parkeri* Cooley were cultured from individuals provided by J.H. Oliver, Georgia Southern University, Statesboro, GA. All stages were fed on white mice, *Mus musculus* and maintained at 25± 2°C, 85± 2% relative humidity, a photoperiod of 15:8 h light:dark and 0.5 h of crepuscular light before and after the light phase.

*2.2. Tissue preparation*

For JH radiosynthesis assays, synganglia, salivary glands, midgut and muscle were assayed separately from *D. variabilis* nymphs at the following developmental times: 3 h after eclosion; 72 h after eclosion (host seeking nymphs); 18 and 72 h after attachment to host (partially fed); and fully engorged (2 d after detachment from the host). Only the synganglia were removed from nymphs 3h after eclosion. Adult female *D. variabilis* were assayed at the following time points: 3 h after eclosion; 72 h after eclosion (host seeking adults); 12, 72 and 168 h after host attachment (partially fed, unmated); and after repletion (mated females, 2 d after detachment from the host). The separate tissues examined from adults were synganglia, salivary gland, midgut, ovary and fat body (from replete only). In addition, backless nymphs (72 h after attachment, partially fed) and adult females (partially fed for 72 h, unmated) were assayed. Backless ticks are those in which the dorsal integument is removed from the idiosoma. This was done by making a single incision along the lateral margin of the alloscutum and scutum followed by a lateral incision across the anterior region of the scutum immediately posterior to the capitulum (connecting the endpoints of the first incision). The dorsal integument including the epidermis was carefully removed in order to

achieve minimum disturbance of the underlying tissues. Synganglia, salivary glands, midgut and muscle dissected from last stadium and adult *O. parkeri* (1-2 h after engorgement) were also assayed for JH biosynthesis.

Ticks were surface sterilized by washing with slow agitation in 0.5% (v/v) sodium perchlorate in a 0.01% (v/v) Tween 20 solution for 5 min, in 10% (w/v) sodium thiosulfate for 45 s and in 70% ethanol for 5 min. Ticks were rinsed with two washes of filter sterilized, deionized water. Tissues were then dissected in filter sterilized *Blattella germanica* saline as described by Kurtti and Brooks (1976). Synganglia (Binnington, 1986) included the retrocerebral organ complex, lateral segmental organs, proximal pedal nerves, periganglionic sheath, part of the esophagus passing through the synganglion, the proximal region of all other nerves to the tick periphera and any attached tracheae. Salivary glands were dissected intact and included all associated acini and salivary ducts. Ovaries included the oocytes and oviducts. Peripheral fat body was obtained by removal of the tracheal trunks from their attachment to the spiracles along with its associated fat body. The midgut was removed with the ventriculus and all diverticula intact. Dorso-ventral muscles were removed from the inner surface of the dorsal and ventral integument.

*2.3. JH biosynthesis studies*

Juvenile hormone radiosynthesis assays were modified from the procedure of Pratt and Tobe, (1974). Tissues were incubated in modified L-15B tick media (Munderloh and Kurtti, 1989) containing L-[*methyl*-$^{3}$H]methionine as the only source of methionine or containing [1-$^{14}$C]acetate with non-radiolabeled methionine. Tritiated methionine was used at a final concentration of 50 μM (71.4 Ci/mmol, New England Nuclear, Wilmington, DE), while [1-$^{14}$C]acetate was used at 19.34nM (51.7 mCi/mmol, Sigma Chemical, St. Louis, MO). The incubation media also included the JH esterase inhibitor, OTFP (3-octylthio-1,1,1-trifluoropropan-2-one), at 0.5 μM and 40 μM farnesoic acid (for the $^{3}$H-methionine media only). Controls

for the $^{3}$H-methionine labeling experiments were five corpora allata per assay from either 5-16 d old, fourth stadium *Diploptera punctata* (Eschscholtz) or fourth stadium *Gromphadorina portentosa* Schaum (supplied by G.L. Holbrook, North Carolina State University, Raleigh). Corpora allata from vitellogenic, female *Periplaneta americana* L. were used as controls in the $^{14}$C-acetate labeling studies. Farnesoic acid was prepared according to the method of Borovsky et al. (1994). OTFP was prepared as described by Hammock et al. (1982, 1984). Tissues were incubated at 25°C for 3 h in 5 μl of media on hexane-rinsed, oven-baked (1h at 100°C) glass coverslips contained in a 24-well tissue culture plate (Falcon 3047, Beckton-Dickinson, Lincoln Park, NJ). The plates were maintained at 100% relative humidity. Ten synganglia were used for each incubation. For salivary gland, gut, ovary, muscle and fat body, ten pieces of approximately the same size each as a single synganglion were used per assay. After incubation, tissues and media were extracted with 100 μl each of water and organic solvent (either hexane or ethyl acetate) by sonication for 15 s, followed by centrifugation at 13,000 x *g* for 10 min at 4°C. No radioactivity remained on the glass coverslip.

*In vivo* radiosynthesis assays were conducted by injecting 2 μl of media containing L-[*methyl*-$^{3}$H]methionine into partially fed (completed slow feeding), unmated and replete, mated (2 d after detachment) *D. variabilis* females. Fourth stadium *D. punctata* (5-16 d old) was used as a positive control. *D. varaibilis* were injected through the ventral integument between coxae I and II *and D. punctata* through the thin, flexible cuticle surrounding coxa II. After a 24 h incubation period at 25°C, the samples were homogenized at room temperature in 3 ml of hexane:water (1:1, v/v) with a Polytron PT10-35 homogenizer fitted with a PT10-ST probe generator (Brinkman, Westburg, NY) followed by centrifugation at 1,830 x *g* at 4°C for 10 min. The hexane phase was removed, concentrated just to dryness under a stream of $N_2$ in a 35°C water bath and subsequently re-dissolved in 100 μl of hexane. For all $^{3}$H-labeling experiments, the

hexane phase was reserved for radio-high performance liquid chromatography (vide infra). Ethyl acetate was the organic solvent used in the $^{14}$C-acetate studies.

*2.4. Synthesis of JH standards*

Juvenile hormone III (JH III) was obtained from Sigma Chemical. Juvenile hormones I and II were purchased from Calbiochem (San Diego, CA). Farnesol was obtained from Aldrich Chemical. Methyl farnesoate (MF) and JH III bisepoxide ($JHB_3$) were synthesized according to the method of Yin et al. (1995). JH III-diol was prepared enzymatically according to the method of Linderman et al. (1995) as well as chemically by the method of Rembold et al. (1980). JH III-acid was prepared by incubating JHIII in 500 µl of 0.5 M NaOH in 50% ethanol for 3 h at 50°C and then isolated from the ethanolic NaOH by three extractions with 1 ml of ethyl acetate:water each (1:1, v/v). With each extraction the ethyl acetate phase was reserved and concentrated under nitrogen. JH III-ethyl (10,11-epoxy-3,7,11-trimethyl-trans, trans-2,6-dodecadienoic acid ethyl ester) was prepared from JH III-acid via diazoethane alkylation. Briefly, 100 mg of 1-ethyl-3-nitro-1-nitrosoguanidine (Aldrich Chemical) was added to 2 ml of 5 M NaOH:diethyl ether (1:1, v/v) and stirred until the mixture ceased to bubble. The yellow diethyl ether layer was removed and combined with 1 ml diethyl ether containing JH III-acid and stirred for 5 min. The ether was evaporated by vacuum centrifugation (Savant Instruments, Hicksville, NY) and the remaining residue dissolved in hexane. After three extractions with 40% aqueous methanol, the hexane phase yielded pure JH III-ethyl.

*2.5. Derivitization of radiolabeled tick products*

Selected radiolabeled products produced by tick tissues were further analyzed for the presence of epoxide or ester groups typical of the insect JHs by chemical and enzymatic derivitization. Ester hydrolysis was conducted with 0.5 M NaOH in 50% aqueous ethanol (Borovsky et al., 1994). Enzymatic epoxide hydration was conducted with microsomes from last stadium, wandering (whole body) cabbage loopers,

*Trichoplusia ni*, as described by Linderman et al. (1995) or chemically by treatment with 0.06% (v/v) perchloric acid in ethyl acetate for 2 h at 25 °C (Rembold et al., 1980). Simultaneous controls were conducted with [10-$^{3}$H(N)]-JHIII (16 Ci/mmol, New England Nuclear). Products were analyzed by radio-HPLC.

*2.6. Radio-HPLC*

Detection of radiolabeled tick products was performed using a Beckman HPLC system (Beckman Instruments Inc., Fullerton, CA) equipped with model 125 pumps, a model 166 UV detector ($A_{214}$) and a model 171 radioactive flow detector. HPLC data were collected and analyzed using the Beckman System Gold® software. Samples from $^{3}$H-methionine labeling studies were chromatographed on an Econosil $C_{18}$ reverse-phase HPLC column (4.6 mm x 25 cm, 5 μm particle size, Alltech Associates, Deerfield, IL) using a linear gradient program. HPLC conditions were as follows: run length 50 min, flow rate 1 ml/min, mobile phase 40% acetonitrile: 60% 5 mM Hepes pH 7.5 to 100% acetonitrile over 45 min with a 5 min hold. The scintillation fluid flow rate was 3.0 ml/min (Beckman Ready-Flow III, Beckman Instruments). Samples from the $^{14}$C-acetate labeling studies were analyzed using a Ultrasphrere $C_{8}$ reverse-phase HPLC column (4.6 mm x 25 cm, 5 μm particle size, Beckman Instruments, Fullerton, CA) under isocratic conditions: run time 75 min, flow rate 1 ml/min, mobile phase of 60% acetonitrile: 40% 5 mM Hepes pH 7.5. Counting efficiency for $^{3}$H and $^{14}$C was 17% and 62%, respectively, with a minimum detection limit of 200 DPM or 1.27 fmol for methyl tritiated JH III.

*2.7. Galleria bioassay*

Bioassays for possible insect JHs in *D. variabilis* were conducted using the *Galleria* cuticle-wax assay (DeLoof and Van Loon, 1980). *Galleria mellonella* L. larvae (Carolina Biological, Burlington, NC) were cultured as larvae until pupation at 30°C (14:10 L:D, 35% RH) on standard diet furnished by the

company. *D. variablis* eggs (10 and 15 d old), unfed larvae (1-5 d after eclosion), unfed nymphs (1-7 d after eclosion) (5 g each), and partially fed, unmated females (completed slow feeding phase)(10 g) were added to $CHCl_3$:methanol:water (1:2:0.8, v/v/v) and immediately homogenized at room temperature with a Polytron PT10-35 homogenizer with a PT10-ST probe generator (Brinkman). Total lipid was extracted with $CHCl_3$ by the method of Bligh and Dyer (1959), concentrated by evaporating the $CHCl_3$ and mixed with paraffin wax in a 1:1 ratio (w/w). The lipid:wax mixture (1 mg) was applied to the dorsum of the thorax where a 1.0 $mm^2$ section of cuticle was surgically removed. The wax was melted with the aid of a warm soldering iron, sealing the wound. Positive controls were pupae treated with 100 pg of JH III (Sigma Chemical) in 1mg of peanut oil:wax (1:1, w/w) and those treated with 1 mg of a mixture of whole body, total lipid from fourth stadium *D. punctata* (5-16 d old) and paraffin wax (1:1, w/w). The *D. puncata* total lipid was prepared from five individuals with the same methods as for the tick samples. The negative control was 1 mg of paraffin wax and peanut oil only. Pupae were examined 5-6 d after treatment. A positive response appeared as a darkened, wrinkled area of cuticle underneath the wax preparation. Adults were examined after eclosion to determine the presence or absence of juvenized cuticle. The lower detection limits for JH I and JH II was 2 pg while that for JH III was 70 pg (DeLoof and Van Loon, 1980).

*2.8. EI GC-MS*

A 1 ml volume of hemolymph from partially fed, virgin *D. variabilis* and replete, mated *D. variabilis* and *O. parkeri* females was collected from severed legs into graduated micro-capillary tubes, immediately expressed into ice-cold acetonitrile and stored at –80°C. Capillaries in which midgut contents appeared were discarded. The hemolymph yield from individual virgin *D. variabilis* ranged from 1 to 10 μl while that from replete *D. variabilis* and *O. parkeri* was 0.5 to 3 μl. Fourth stadium *Diploptera punctata* (100 μl) hemolymph was collected and stored in the same manner as tick hemolymph. Fifteen nanograms of JH III-

ethyl was added to tick hemolymph/acetonitrile as an internal standard prior to centrifugation at 7,200 x *g* (4°C) for 5 min. The acetonitrile supernatant was decanted and concentrated to 100 µl by vacuum centrifugation at room temperature. The acetonitrile extracts were purified by $C_{18}$ reverse phase HPLC using the method described above. Eluant at 1 min prior to and after the exact retention times for JH III-diol, $JHB_3$, JH III, JH III-ethyl, farnesol, JH II, JH I and MF (in order of elution) were collected separately, and NaCl added to saturation and mixed vigorously. Samples were then centrifuged at 1,830 x *g* for 5 min (room temp) followed by removal of the upper acetonitrile phase. The acetonitrile was evaporated by vacuum centrifugation at room temperature and stored at –80°C. Prior to mass spectrometry, the compounds collected by HPLC (except fractions corresponding to $JHB_3$, MF and farnesol) were converted to deuterated methoxyhydrin derivatives ($d_3$-MH) based on the methods modified from Bergot et al. (1981) and Brindle et al. (1987). To the residue from HPLC purification, 50 µl of $CD_3OD$ (methyl-$d_3$-alcohol-d, 99.8 atom % D, Sigma Chemical) was added, followed by 50 µl of 5% trifluoroacetic acid (Sigma Chemical) in $CD_3OD$. The vial containing this solution was sealed with a Teflon lined screw cap and heated for 15 min at 65°C, followed by concentration via vacuum centrifugation. The residue was dissolved in 100 µl of hexane and applied to an alumina (Sigma Chemical) column 0.6 x 4.0 cm in size (activity grade VI) pre-rinsed with 2 ml of 30% diethyl ether in hexane. After sample application, the column was eluted with 2 ml of 30% diethyl ether in hexane to remove any underivitized compounds. Methoxyhydrin derivatives were eluted from the column with 3 ml ethyl acetate: hexane (1:1, v/v) and concentrated by vacuum centrifugation at room temperature.

Analyses were carried out on a Hewlett-Packard Model 6870 GC-MS using a Model 5973 mass selective detector (MSD). The GC was equipped with a HP-5MS (5% diphenyl-95% dimethylsiloxane) capillary column 30 m in length, 0.25 µm film thickness and 0.25 mm inside diameter. The

chromatographic conditions were as follows: initial temperature 150°C, hold for 2 min, ramp at 10°C/min to 270°C and hold for 5 min; helium carrier flow 1.5 ml/min; and injector temperature 250°C in splitless mode. The MS source and quadrapole temperatures were set to 230 and 150°C, respectively. Chromatographic data were collected in scan and selective ion monitoring (SIM) modes. In scan mode, the total ion chromatogram was constructed from a range of 40-300 AMU. The following selective ion monitoring was used: m/z 76 and 225 for JHIII-$d_3$-MH; m/z 76 and 239 for JHIII ethyl-$d_3$-MH; m/z 90 and 225 for JHII-$d_3$-MH; m/z 90 and 239 for JHI-$d_3$-MH; m/z 90 and 253 for JH0-$d_3$-MH; 69, 84 and 136 for farnesol; 69, 114, and 121 for MF; and 71, 111 and 155 for $JHB_3$. Tick hemolymph was not examined for the presence of 4-methyl-JH I (iso-JH 0) by GC-MS. The MS sensitivity was determined to be 1.6 pg in scan mode between 40-300 AMU, while in SIM mode it was 750 fg when monitoring fragments at m/z 76 and 225.

*2.9. Synganglia transplantation*

Synganglia from two engorged, next to last stadium, nymphal *O. parkeri* were dissected as described earlier and immediately transplanted into a single last stadium, nymphal *O. pakeri* (2 synganglia per recipient). The recipients were partially blood fed 24 h prior to transplantation. The synganglia were implanted through an opening in coxa III (after removal of the leg) using a fire polished 1 μl microcapillary to deposit the synganglia into the hemocoel of the recepient. Unoperated controls received no donated synganglia while sham-operated controls received two pieces of salivary gland approximately the same size as two synganglia. The entry wound was sealed with only enough New Skin® liquid bandage (Medtech Laboratories, Inc., Jackson, WY) to cover the wound. The ticks in the treatment and controls were allowed to feed *ad libitum* 7-15 d after transplantation.

## 3. Results

### *3.1 JH biosynthesis in ticks*

Nymphal and adult *D. variabilis* and *O. parkeri* were examined for their ability to synthesize JH III and methyl farnesoate (MF) using a modification of the Pratt and Tobe (1974) assay for JH biosynthesis. In these experiments, synthesis of [*methyl*-$^3$H]-JH III and [*methyl*-$^3$H]-MF either *de novo* or from farnesoic acid (which was added to the incubation medium) could be detected simultaneously. The lower limit of detection for [*methyl*-$^3$H]-JH III and -MF in these studies was 1.27 fmol 10-synganglia$^{-1}$ 3-h$^{-1}$. Synganglia (including the lateral segmental organs) from nymphal and adult *D. variabilis* or *O. parkeri* at numerous stages after eclosion, during and after blood feeding and at various stages during reproduction including the period of vitellogenesis (Table 1) failed to produce [*methyl*-$^3$H]-products that co-chromatographed with JH III diol, JH III bis-epoxide, JH III, JH II, JH I or MF. Typical results are shown in Fig. 1 for synganglia from *D. variabilis* nymphs (engorged, 2 d after detachment) and adults (replete female, mated, 2 d after detachment) and from *O. parkeri*, nymphs and adult females (engorged, 1-2 h after detachment). The incubation medium in these studies included OTFP, which is a potent JH esterase inhibitor; under the conditions of our modified Pratt and Tobe assay, there was no detectable JH esterase activity (data not shown). A radiolabeled product from synganglia was detected at a rentention time of approximately 17 min for both nymphal and adult *D. variabilis* but not *O. parkeri* (Fig. 1). This product was reported earlier as indistinguishable from the JH III bis-epoxide by thin layer chromatography (Roe et al., 1993). In comparison to the results with synganglia, corpora allata from fourth stadium *P. americana* and *G. portentosa*, under identical experimental conditions produced JH III (Fig. 1).

Other tissues were examined either in isolation or in combination and both *in vitro* and *in vivo* in

order to identify a possible source for a tick JH. Midgut, ovary, fat body, salivary gland and muscle assayed from nymphyl and adult *D. variabilis* and *O. parkeri* after eclosion, at varying times during and after feeding and during vitellogenesis (Table 1) again failed to produce any [*methyl*-$^3$H]-products that co-chromatographed with JH III diol, JH III bis-epoxide, JH III, JH II, JH I or MF as was also the case for synganglia. Typical results are shown in Fig. 2 for replete, mated *D. variabilis* females (2 d after detachment). Not only did individual tissues not produce JH, but partially fed backless nymphal (72 h after attachment to the host), engorged backless nymphal (2 d after deteachment), and partially fed backless adult female (72 h after attachment, unmated) *D. variabilis* (Table 1) also failed to produce any insect JH (data not shown). L-[*methyl*-$^3$H]methionine incubation media was injected into partially fed (unmated) and replete, mated (2 d after detachment) *D. variabilis* females. In these experiments as well, no JH biosynthesis was detected by radio-HPLC while simultaneous experiments with fourth stadium *D. punctata* produced JH III and MF(data not shown).

Both synganglia as well as the other individual tissues examined (midgut, ovary, fat body, salivary gland and muscle) in the modified Pratt and Tobe assay produced a radiolabeled product with a retention time of approximately 17 min (Figs. 1 and 2). This product did not co-chromatograph with our known JH standards and since it was produced by all tissues and at all stages of development that were examined in *D. variabilis* only, the product did not appear to be a homolog of JH. In earlier studies by this laboratory (Roe et al., 1993), it was noted that this product co-chromatographed on thin layer chromatography with the bis-epoxide of JH III. When this product produced by the synganglia of partially fed, virgin *D. variabilis* females was subjected to base hydrolysis, its retention time on HPLC was not changed and the tritium label was retained (Fig. 3). In control experiments, the [*methyl*-$^3$H] ester of JH III produced by *D. punctata* corpora allata was also isolated by identical procedures; base hydrolysis of this isolate and of (10-$^3$H)-JH III

removed 100% of the methyl ester resulting in no detectable JH III or (10-$^{3}$H)-JH acid, respectively, as determined by radio-HPLC. Perchloric acid treatment (Rembold *et al*, 1980)(Fig. 3) or incubation of the synganlion product with microsomal epoxide hydrolase as described by Linderman *et al.*(1995)(data not shown) again did not change the retention time of the synganglion product while these same experiments with (10-$^{3}$H)-JH III and the [*methyl*-$^{3}$H] ester of JH III produced by the cockroach CA resulted in JH diol. These studies *in toto* demonstrated that the common product produced by the tissues of *D. variabilis* throughout nymphal and adult development did not have an ester or epoxide moiety common to the insect JHs.

The above biosynthesis experiments only measured methyl farnesoate and JH biosynthesis. Synthesis of farnesol in addition to methyl farnesoate and JH was examined by incubating synganglia from *D. variabilis* partially fed virgin and replete, mated females (4 d after detachment), with [1-$^{14}$C]acetate. In these experiments, no farnesol, methyl farnsoate or JH was detected while corpora allata from vitellogenic, female *P. americana* incubated under identical conditions produced these products (Fig. 4).

*3.2 Tick juvenizing activity in the Galleria bioassay*

No *in vitro* or *in vivo* JH biosyntheis could be detected in nymphs and adults of both *D. variabilis* or *O. parkeri*. To detect the presence of any lipids from any of the stages of tick development that might demonstrate insect juvenizing acivity, 10 and 15 d old eggs, unfed larvae, unfed nymphs and partially fed adult females (completed slow feeding phase) of *D. variabilis* were assayed using the *Galleria* pupal cuticle bioassay (DeLoof and Van Loon, 1980). A positive juvenizing response in this bioassay results in newly formed cuticle in pharate adults exhibiting pupal characteristics (i.e. darkened wrinkled cuticle). The application of JHIII standard and lipid extracts from fourth stadium *D. punctata* produced 72.7% and 100% positive responses (Table 2). In contrast, postive responses produced by lipid extracts from tick eggs,

larvae, nymphs and adults was less than are equal to that of the negative (no JH III) control. The detection limits for eggs, larvae and nymphs were 28 pg JH I, 28 pg JH II and 980 pg JH III per g tissue. For adults, the detection limits were 116 pg JH I, 116 pg JH II and 4,069 pg JH III per g tissue or 7.25 pg JH I, 7.25 pg JH II and 253 pg JH III per tick. No insect juvenizing activity was detectable in undiluted, total lipid from *D. variabilis*.

*3.3 Tick juvenizing activity*

Transplantation experiments were conducted to determine whether there is a tick juvenizing factor in the synganglion that might inhibit tick development. Two synganglia from nymphal, blood fed, next to last instars were transplanted (Cox, 1960) into blood fed last instars of *O. parkeri.* These ticks molted normally to the adult stage similar to the controls (Table 3) and developed normally through 12 wk after surgery. At the conclusion of the experiment, the ticks were dissected; the transplanted synganglia after 12 wk appeared to be normal with no indication of degeneration or encapsulation.

*3.4 EI GC-MS identification of JH in ticks*

EI GC-MS analyses were conducted on hemolymph from partially fed, unmated and replete, mated *D. variabilis* and on replete, mated *O. parkeri* (Figs. 5 and 6). The internal standard, JHIII-ethyl-$d_3$-methoxyhydrin (m/z 76 and 239) was detected in these assays at a retention time of 10.32 min (Fig. 5). No JH I, JH II, JH III, JH III bis-epoxide, farnesol or methyl farnesoate was detected. In comparison, JHIII-$d_3$-methoxyhydrin (m/z 76 and 225; data not shown) was detected in the hemolymph of fourth stadium *D. punctata*. In the tick samples analyzed, a prominent peak with the same retention time as JHIII-$d_3$-methoxyhydrin (9.75 min) was observed (Fig. 5). The EI MS spectrum for this peak is shown in Fig. 6. By comparison with known spectra using the HP ChemStation® MS spectral library (Hewlett-Packard), the compound eluting from the GC at 9.75 min produced a >95% match with $\Delta^9$-oleic acid methyl ester. None

of the tick samples were shown to contain ions diagnostic for any of the JHs, farnesol, or methyl farnesoate. The MS sensitivity was 1.6 pg in the scan mode from 40-300 AMU and 750 fg in the SIM mode for fragments at m/z 76 and 225.

## 4. Discussion

### *4.1 Role of JH in arthropod development*

Our understanding of the hormonal regulation of molting, metamorphosis and reproduction is highly advanced in the insects. Juvenile hormone in insects serves as a *status quo* hormone (Riddiford, 1996a,b). Increasing ecdysteroid levels in the presence of JH initiates a larval-larval molt, and ecdysteroids in the absence of JH initiates metamorphosis. Juvenile hormone so far is only found in the Insecta. Methyl farnesoate (the immediate precursor to JH III) promotes molting and reduces the intermolt period, but demonstrates no *status quo* activity in the Crustacea (Smith and Sedlmeier, 1990; Charmantier *et al.*, 1991; Cusson *et al.*, 1991; Chang *et al.*, 1993; Chang, 1993; Homola and Chang, 1997). In insects and Crustacea, JH and methyl farnesoate, respectively, control vitellogenesis by apparent similar mechanisms. The general assumption for many years has been that ticks use JH like insects to regulate nymphal-adult metamorphosis and yolk deposition (reviewed by Obenchain and Galun, 1982; Sonenshine, 1991). Based on the abundance of smooth endoplasmic reticulum, the putative site for JH biosynthesis in ticks was the lateral segmental organs.

### *4.2 Role of JH in tick molting and metamorphosis*

There have been several studies on the effect of JH, JH analog and anti-JH (precocene) treatments on nymphal molting and metamorphosis in ticks (McDanial and Oliver, 1978; Leahy and Booth, 1980; Khalil *et al.*, 1984; Abdelmonem *et al.*, 1986). These experiments have been conducted by different

laboratories on three different tick species. Leahy and Booth (1980) could not rescue with JH the precocene induced mortality associated with nymphal-adult molting in *Argas persicus*, and JH or JH analog treatments did not delay metamorphosis or induce supernumery molting in other studies (McDaniel and Oliver, 1978; Khalil *et al.*, 1984; Abdelmonem *et al.*, 1986). An exception was the findings of Khalil *et al.* (1984), where JH I delayed nymphal molting 9-31% but did not produce supernumery molts when the application was made on the day of engorgement in the camel tick, *Hyalomma dromadarii*. No delay was noted at any of the other treatment times after attachment or engorgement.

Literature evidence for a *status quo* function for JH in nymphal ticks is minimal. In addition, there is a surprising lack of research on obtaining direct chemical proof for JH in nymphal ticks. For these reasons, extensive experiments were conducted to investigate JH biosynthesis by *D. variabilis* and *O. parkeri* nymphs. In these studies, synganglia were incubated with farnesoic acid and high specific-activity L-[*methyl*-$^{3}$H]methionine, so that the only steps required for JH production by the tick were formation of the methyl ester and synthesis of the JH C10,11 epoxide. Numerous developmental stages were examined in unfed, partially fed and engorged nymphs (Table 1) with a lower detection limit of 1.27 fmol for JH or methyl farnesoate 3-h$^{-1}$ for 10 glands. The synganglion dissection included the lateral segmental organs, the putative site for JH biosynthesis. No JH I, JH II, JHIII, JH III bisepoxide, or methyl farnesoate was detected by radio-HPLC while corpora allata from two cockroach species under identical experimental conditions produced JH as expected. Selected experiments were also conducted using L-[*methyl*-$^{3}$H]methionine but no farnesoic acid with the same results, no synthesis of JH (data not shown). During the course of these in vitro incubations, the synganglia of *D. variabilis* appeared normal in shape and color and produced in a time-dependent fashion, a hexane soluble radiolabeled product that failed to co-chromatograph with JH standards. This product was synthesized by all tick tissues examined (synganglia, midgut, muscle and

salivary gland) and apparently lacked an epoxide and methyl ester. Synganglia, midgut, muscle and salivary glands alone or together (backless ticks) in *D. variabilis* and in the soft tick, *O. parkeri*, also failed to synthesize JH or methyl farnesoate. *O. parkeri* was chosen as a model in our studies because Pound and Oliver (1979) hypothesized that JH regulated egg maturation in this species (discussed in more detail later).

In both hard and soft ticks, there was no evidence of JH biosynthesis either *de novo* or from farnesoic acid in isolated tissues and tissues in combination (backless ticks) throughout nymphal development. Since JH biosynthesis was not detected in nymphal ticks, *Galleria* pupal cuticle bioassays of whole-body tick extracts and intra-species synganglia transplantation experiments were conducted to assay for juvenilizing activity in general. The only assumptions made in these experiments were that the tick juvenilizing agent was lipid soluble or the origin was the synganglion. Potential juvenilizing activity was also tested using both an insect and tick system. No positive responses above background were detected in lipid extracts from eggs, larvae, nymphs and adults of *D. variabilis* using the *Galleria* assay while JH III and cockroach controls were positive for juvenilizing activity. Synganglia from next to last nymphal instars of *O. parkeri* also produced no detectable juvenilizing agents when transplanted into last instars.

Biosynthesis, bioassay and transplantation experiments do not support a role for JH in the regulation of tick molting and metamorphosis, or the presence of a tick *status quo* hormone. The lack of evidence for a *status quo* hormone in ticks is similar to that found in the Crustacea (Homola and Chang, 1997).

*4.3 Role of JH in tick reproduction*

The strongest evidence for JH in ticks and for a possible functional role for JH in tick reproduction was in the study by Pound and Oliver (1979). The application of the anti-JH, precocene II, clearly inhibited

egg production in *O. parkeri*. The subsequent treatment with 1 μg of JH III rescued the anti-JH effect and egg production resumed, suggesting that the reproductive hormone of ticks was juvenile hormone. However, in the experiments of Pound and Oliver (1979), JH doses greater than 1 μg decreased egg production and this decrease was dose-dependent. If the exogenous JH treatment in these experiments replaced endogenous tick JH eliminated by the precocene treatment, egg production would be expected to remain the same or increase as the JH dose is increased; however, the opposite was found. The results although suggestive for a functional role for JH in ticks, were equivocal. When these studies were conducted in three other tick species, *A. persicus* (Leahy and Booth, 1980), *Boophilis microplus* (Connat, 1988) and another *Ornithodoros* species, *O. moubata*, (Connat and Nepa, 1990), no JH rescue and resumption of normal egg development occurred for precocene or fluoromevalonate treated ticks, suggesting that juvenile hormone was not important in egg maturation.

The most convincing evidence suggesting that JH was not involved in the regulation of vitellogenesis was provided by Chinzei and Taylor (1990), Taylor *et al.* (1991), and Chinzei *et al.*, (1991) in *O. parkeri* and *O. moubata*. The topical application in acetone and the injection in mineral oil of JH homologues, JH acid and JH analogues failed to induce vitellogenesis while the pyrethroid, cypermethrin, acted as a positive control. Khalil et al. (1984) also was unable to increase egg production with JH I while Connat *et al.* (1983) found that JH and JH analogues increased egg laying. Dees *et al.* (1982), Khalil *et al.* (1984) and Abdelmonem *et al.* (1986) found that JH, JH analogues and precocenes had no effect on sexual attraction, and Solomon and Evans (1977), Mansingh and Rawlins (1977) and Teel et al. (1996) found that JH analogues inhibited vitellogenesis or oviposition. Recall that Pound and Oliver (1979) also found that high levels of JH III inhibited egg production in their precocene rescue experiments.

It is apparent that the literature on the role of juvenile hormone in the regulation of tick reproduction

is contradictory. In order to address this question, studies were conducted to identify JH in adult ticks. JH biosynthesis assays were conducted with L-[*methyl*-$^{3}$H]methionine and farnesoic acid in individual tissues and at numerous developmental times in *D. variabilis* (Table 1). Like in nymphs, no JH I, JH II, JH III, JH III bisepoxide, or methyl farnesoate was detected by radio-HPLC even though a hexane soluble product common to all tissues was found. This common product was unaffected by treatment with acid, base, or epoxide hydrolase and did not co-chromatograph with know JH standards as was previously discussed for nymphs. Backless, 72 h old partially fed *D. variabilis* and synganglia, salivary glands, midgut, and muscle from engorged *O. parkeri* also failed to produce JH or methyl farnesoate. In addition, synganglia from partially-fed virgin and replete *D. variabilis* incubated with [1-$^{14}$C]acetate failed to demonstrate the synthesis of farnesol, methyl farnesoate of JH in contrast to positive insect controls. JH was also not produced when L-[*methyl*-$^{3}$H]methionine was injected into intact engorged, unmated or replete, mated *D. variabilis* while the same experiments in fourth stadium *D. punctata* were positive for JH III.

There was no evidence of farnesol, methyl farnesoate or JH biosynthesis in adult hard and soft ticks. These findings were in agreement with the absence of juvenilizing activity in eggs, unfed larvae, unfed nymphs and partially fed, unmated females of *D. variabilis* as measured by the *Galleria* pupal cuticle bioassay, and the lack of JH in the hemolymph of partially fed virgin and replete, mated *D. variabilis* and engorged *O. parkeri*, as determined by EI GC-MS.

*4.4 Summary*

Our current studies suggest that ticks do not synthesize or have measurable amounts of farnesol, methyl farnesoate, JH I, JH II, JH III, or JH III bisepoxide and apparently do not use these hormones as a "status quo" hormone to regulate nymphal-adult metamorphosis or to control vitellogenesis. No insect juvenilizing activity could be found in tick eggs and larvae as well. The literature reports of the effects of

anti-JHs, JH, and JH analogues on tick development and the report of the JH rescue of precocene inhibition of egg production is contradictory to these findings and are unexplained. However, it should be noted that JH rescue of precocene inhibition of growth and JH effects on behavior in invertebrates outside the Arthropoda have been previously reported (Fodor *et al.*, 1982, Davey, 1988, Biggers and Laufer, 1996). Terpenoids have also been found in the mites, *Suidasia medanensis* (farnesal, Leal *et al.*, 1989) and *Tetranychus urticae* (gerniol, Regev and Cone 1975, 1976a,b) where they are used as pheromones; however, their origin of synthesis was not determined. Chinzei and Taylor (1990, 1994), Chinzei *et al.* (1992), Taylor and Chinzei (1999), Schriefer (1991), Coons (personal communication), Neese *et al.* (unpublished data) have also demonstrated that synganlgion factors and ecdysteroids affect yolk protein biosynthesis in ticks. Investigating the possible role of these factors in the regulation of metamorphosis and reproduction will be essential to our understanding of the biology of this important Arthropod group and likely the Acari in general. However, based on this current study, there is no direct evidence for the presence of JH in ticks, and the hypothesis that the insect JHs are important factors in the regulation of metamorphosis and reproduction in ticks does not seem likely.

**Acknowledgements**

The authors wish to thank Nanda Gudderra, Woody Bailey, Robert Johns, Doug Clemons and Dr. Glenn Holbrook for their assistance. This work was supported by grants from the Public Health Service-National Institutes of Health (1 RO1 Al 36257) to R.M.R, C.S.A. and D.E.S., from the North Carolina Biotechnology Center ( 9603-IDG-1013) to R.M.R. and C.S.A., and from the North Carolina Agricultural Research Service to R.M.R. (NC05936) and C.S.A. (NC06300)

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Table 1. Stage, developmental time, feeding status and tissues of *Dermacentor variablis* (*D. v.*) and *Omithodoros parkeri* (*O. p.*) assayed with L-[*methyl*-$^{3}$H]methionine and farnesoic acid.

| SPECIES | STAGE | DEVELOPMENTAL TIME | FEEDING STATUS | TISSUE USED IN ASSAY[a] |
|---|---|---|---|---|
| *D. v.* | Nymph | 3 h after eclosion | UF | SYN |
| | | 72 h after eclosion (host seeking) | UF | SYN, SG, G, M |
| | | 18 h after attachment to host | PF | SYN, SG, G, M |
| | | 72 h after attachment to host | PF | SYN, SG, G, M |
| | | 72 h after attachment to host | PF | Backless [b] |
| | | 2 d after detachment | NG | SYN, SG, G, M |
| | | 2 d after detachment | NG | Backless |
| | Adult FM | 3 h after eclosion | UF | SYN, SG, G, OV |
| | | 72 h after eclosion (host seeking) | UF | SYN, SG, G, OV |
| | | 12 h after host attachment (unmated) | PF | SYN, SG, G, OV |
| | | 72 h after host attachment (unmated) | PF | SYN, SG, G, OV |
| | | 72 h after host attachment (unmated) | PF | Backless |
| | | 168 h after host attachment (unmated) | Completed SF, PF | SYN, SG, G, OV |
| | | 2 d after detachment (replete, mated) | Completed FF, NG | SYN, SG, G, OV, FB |
| *O. p.* | Last instar (nymph) | 1-2 h after detachment | NG | SYN, SG, G, M |
| | Adult FM | 1-2 h after detachment | NG | SYN, SG, G, M |

[a]FB, Fat Body; FF, Fast Feeding; FM, Female; G, Gut; M, Muscle; NG, Engorged; OV, Ovary; PF, Partially Fed; SF, Slow Feeding; SG, Salivary Gland; SYN, Synganglia; UF, Unfed.
[B]Backless ticks refer to those in which the dorsal integument is removed and the remaining tissues are incubated *in situ.*

Table 2. *Galleria* pupal cuticle bioassay for juvenile hormone at different stages of tick development in the American dog tick, *Dermacentor variabilis*..

| TREATMENT | PUPAE EXHIBITING POSITIVE RESPONSE [g] | PUPAE TREATED | % PUPAE EXHIBITING POSITIVE RESPONSE |
|---|---|---|---|
| Untreated control [a] | 1 | 10 | 10.0 |
| 100 pg JH III [b] | 8 | 11 | 72.7 |
| *D. punctata* lipid extract [c] | 10 | 10 | 100 |
| *D. variabilis* 10 d egg | 0 | 10 | 0 |
| *D. variabilis* 15 d egg | 0 | 13 | 0 |
| *D. variabilis* larvae [d] | 0 | 11 | 0 |
| *D. variabilis* nymph[e] | 1 | 14 | 7.1 |
| *D. variabilis* adult [f] | 1 | 10 | 10.0 |

[a] Untreated controls were peanut oil combined with wax (1:1, w/w). JH)III control was 100pg JHIII in peanut oil combined with wax (1:1, w/w).
[c] Lipid extract (no solvent carrier) from 5-16 d old, 4th stadium *Diploptera punctata* combined with wax (1:1, w/w).
[d] Lipid extract (no solvent carrier) from unfed larvae combined with wax (1:1, w/w).
[e] Lipid extract (no solvent carrier) from unfed nymphs combined with wax (1:1, w/w).
[f] Lipid extract (no solvent carrier) from unmated females completing slow feeding phase combined with wax (1:1, w/w).
[g] Darkened wrinkled cuticle in wound 5-6 d after pupation (DeLoof and Van Loon, 1980).

Table 3. Synganglia transplantation in nymphal *Ornithodoros parkeri*.

| DONOR [a] | RECIPIENT | TICKS TREATED | NORMAL EDYSIS TO ADULT | TICK MORTALITY |
|---|---|---|---|---|
| Next to last instar | Last instar | 17 | 16 | 1 |
| Control | Last instar | 13 | 13 | 0 |
| Unoperated control | Last instar | 11 | 11 | 0 |

[a] Two synganglia from nymhal *O. parkeri* in the next to last stadium were transplanted into a single last instar 24 h after engorgement for each replicate. The controls received salivary gland tissue of the same apparent size as two synganglia.

## FIGURE CAPTIONS

Fig. 1. Radio-HPLC analysis of $^{3}$H-labeled products synthesized *in vitro* by the synganglia *of* engorged nymphs (2 d after detachment) and replete (mated) adult females (2 d after detachment) of *Dermacentor variabilis* (*D.v.*) and engorged last stadium nymphs and adult females (1-2 h after detachment) of *Ornithodoros parkeri* (*O.p.*). The positive controls were corpora allata from fourth stadium *Diploptera punctata* (*D.p.*) and *Gromphadorina portentosa* (*G.p.*). Incubations were conducted in media containing L-[*methyl*-$^{3}$H]methionine and farnesoic acid. Retention times of juvenile hormone I (JH I), juvenile hormone II (JH II), juvenile hormone III (JH III), juvenile hormone III bisepoxide ($JHB_3$), juvenile hormone III diol ($JHD_3$) and methyl farnesoate (MF) are indicated. The retention time is in min.

Fig. 2. Radio-HPLC of $^{3}$H-labeled products synthesized *in vitro* by midgut, ovary, synganglia, fat body, salivary gland and muscle from replete, mated adult females (2 d after detachment) of *Dermacentor variabilis*. Incubations were conducted in media containing L-[*methyl*-$^{3}$H]methionine and farnesoic acid. Retention times of juvenile hormone I (JH I), juvenile hormone II (JH II), juvenile hormone III (JH III), juvenile hormone III bisepoxide ($JHB_3$), juvenile hormone III diol ($JHD_3$) and methyl farnesoate (MF) are indicated. The retention time is in min.

Fig. 3. Radio-HPLC of $^{3}$H-labeled products synthesized *in vitro* by synganglia from replete, mated adult females of *Dermacentor variabilis* (2 d after detachment) subjected to base hydrolysis using 0.5 M sodium hydroxide or acid hydrolysis with 0.06% perchloric acid. These results are compared to the untreated control. Synganglion incubations were conducted in media containing L-[*methyl*-$^{3}$H]methionine and farnesoic acid. Retention times of juvenile hormone III (JH III), juvenile hormone III acid ($JHA_3$) and juvenile

hormone III diol ($JHD_3$) are indicated. The retention time is in min.

Fig. 4. Radio-HPLC of $^{14}C$-labeled products synthesized *in vitro* by synganglia from adult unmated females (168 h after host attachment) and replete, mated adult females of *Dermacentor variabilis* (*D.v.*) (3 d after detachment) and by corpora allata from vitellogenic *Periplaneta americana* (*P.a.*) females. Incubations were conducted in media [1-$^{14}C$]acetate and no L-[*methyl*-$^{3}H$]methionine or farnesoic acid. Retention times of farnesol, juvenile hormone III (JH III) and methyl farnesoate (MF) are indicated. The retention time is in min.

Fig. 5. Total ion chromatogram of hemolymph extracts from adult unmated females (168 h after host attachent) and replete, mated adult females (1-2 d after detachment) of *Dermacentor variabilis* (*D.v.*) and fully engorged adult females of *Ornithodoros parkeri* (*O.p.*)(1 d after detachment). The samples were subjected to trifluoroacetic acid:methyl-$d_3$-alcohol-d derivitization. Retention times of juvenile hormone (JH) I, II, III, and III ethyl-deuterated-methoxyhydrins ($d_3$-MH) are indicated in the table at the top of the figure. The retention time is in min.

Figure 6. Electron impact mass spectrum of the GC eluant (9.75 min) obtained from the hemolymph of replete (mated) adult females (1-2 d after detachment) of *Dermacentor variabilis* (*D.v.* replete mated) (Fig. 5) with the same retention time as $d_3$-MH (the JHIII-$d_3$-methoxyhydrin).