of the CA, the following changes in the post-emergence reproductive development of the fire ant are initiated: wing casting, flight muscle histolysis, and oocyte development.

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2 G.P. Markin, H.L. Collins and J.H. Dillier, Ann. ent. Soc. Am. *65*, 1053 (1972).
3 P.M. Toom, E. Cupp, C.P. Johnson and I. Griffith, J. Insect Physiol. *22*, 217 (1976a).
4 P.M. Toom, E. Cupp and C.P. Johnson, Insect Biochem. *6*, 327 (1976b).
5 J.F. Barker, Gen. Comp. Endocr. *35*, 234 (1978).
6 G.L. Humason, Animal Tissue Techniques. W.H. Freeman, San Francisco 1967.

Ecdysteroids in *Limulus* larvae[1]

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Summary. Free ecdysteroids were extracted from intermolt and premolt larvae of *Limulus polyphemus* in the first post-hatch stage, purified by TLC and HRLC, and assayed by RIA and the *Limulus* bioassay. 20-hydroxyecdysone appears to be a principal ecdysteroid and occurs at least 3 times higher a concentration in premolt vs intermolt animals.

Ecdysteroids have been identified in many species of crustaceans and insects but little is known about these compounds in other classes of the phylum Arthropoda. Injected ecdysteroids are effective in stimulating molting in representative members of the major classes of arthropods[3,4], yet the majority of the work on extracting and identifying these compounds has been performed on crustaceans and insects; there are, however, many references to the presumption that 20-hydroxyecdysone(ecdysterone) is a general arthropod molting hormone[5]. This compound is very effective as a molt-promoting agent in *Limulus* larvae, and here we report evidence for its occurrence in these animals.

Material and methods. Larvae were collected on Shackleford Banks near the Duke Marine Laboratory in 2 batches. 1 batch was freshly hatched and the larvae were cultured at 25 °C in large glass bowls until they were in premolt, then frozen until extraction. A 2nd batch was collected as larvae hatched in the sand substrate, cultured for 2 days at 25 °C (this produces larvae in intermolt, less than half way through the molt cycle), then frozen. Extraction was based on the method of Karlson and Shaaya[6]. These crude extracts were further purified by 1 of the following 2 methods. a) The 1st consisted of extensive clean-up by TLC on silica gel G plates (Analtech) using 3 solvent systems ($CHCl_3$:EtOH, 4:1 followed by EtOAc: MeOH:NH_4OH:H_2O, 10:2:1:1 and $CHCl_3$:MeOH: HOAc, 4:1:0.05) and 2 ecdysteroids (20-hydroxyecdysone and ponasterone A) as standards. After the 1st system 4 prominent bands in the exdysteroid zone were eluted, re-chromatographed in the 2nd system where most of the polar material was separated from the ecdysteroids. The narrow ecdysteroid zone was eluted and re-chromatographed in the 3rd solvent system. 2 bands were eluted from the plates for assay; one that co-chromatographed with 20-hydroxyecdysone (R_f 0.36) and one conspicuous band (R_f 0.52) chromatographing below ponasterone A (R_f 0.60). b) The 2nd method consisted of an initial clean-up by preparative high resolution liquid chromatography (HRLC) using a Poragel PN column and 50% MeOH:H_2O as the solvent system at a flow rate of 0.6 ml/min. Retention times for 20-hydroxyecdysone, ecdysone and the ponasterone A peak were 25–47, 47–75, and 180 min respectively. 9 fractions were collected over a period of 4 h. These were subjected to TLC using the $CHCl_3$:EtOH, 4:1 solvent system. The band co-chromatographing with pure 20-hydroxyecdysone (R_f 0.24) was eluted for assay. Ecdysteroids were detected by a radioimmune assay and the *Limulus* bioassay[4,8].

Results. The RIA and bioassay results are nearly identical for portions of the extract that, in the final purification step, co-chromatographed with 20-hydroxyecdysone (table). The results were also alike when final products from the 2 purification methods were compared. The only known ecdysteroid that at least partially co-chromatographs with 20-hydroxyecdysone in the final purification steps used is inokosterone. Inokosterone is, however, much less reactive in RIA (4×) than bioassay when compared to 20-hydroxyecdysone, and, if it were present in anything but small quantities, the results from RIA and bioassay would not be similar. Therefore we conclude that 20-hydroxyecdysone is the ecdysteroid we extracted.

The unknown band which resulted from the extract subjected to extensive TLC clean-up showed low activity in the RIA test but very high activity in the bioassay (table). Some pure ecdysteroids, such as makisterone A and cyasterone, give the same kind of effect in our detection methods. The unknown is, however, neither of these compounds. We have no details on the nature of this unknown material, except that it chromatographs between ecdysone and ponasterone A on TLC in a $CHCl_3$:MeOH, 4:1 system and is several hundred times more active in the bioassay than in the RIA. Data in the table also show approximately a 3 times greater concentration of 20-hydroxyecdysone in the premolt than during the intermolt stage in both RIA and bioassay. This finding is in agreement with data on injections of exogenous 20-hydroxyecdysone where only about 100 pg will accelerate molting in 50% of the experimental animals in late intermolt-early premolt as opposed to 500 pg or more for mid-intermolt animals. Changing cell

Quantification of free ecdysteroids in *Limulus* larvae at 2 different stages in the molt cycle

Ecdysteroid	Stage	Detection method	20-hydroxy-ecdysone equivalents pg/larva	ng/g
20-Hydroxyecdysone	Intermolt	RIA	47	9.4
		Bioassay	53	10.6
	Premolt	RIA	135	27.0
		Bioassay	179	35.8
Less polar unknown	Premolt	RIA	32	6.4
		Bioassay	946	189

sensitivities and progress in the 'programmed development' of the larvae may have a role in this physiological effect, but it seems highly probable that increased titres of free ecdysteroids also have a role.

Tritiated ecdysone was injected into premolt larvae and the extract assayed by radio TLC. The ^{3}H-ecdysone is efficiently converted to ^{3}H-20-hydroxyecdysone during an 8–14-h incubation period. Additional confirmation of the product was obtained by acetylation of the eluted ^{3}H-20-hydroxyecdysone by acetic acid-pyridine. TLC of the acetylation products gave a nice fingerprint of the 4 20-hydroxyecdysone acetates.

Discussion. The identification of 20-hydroxyecdysone in *Limulus polyphemus* larvae is based upon purification in TLC and HRLC systems and detection of the products by RIA and bioassay. The data from these experiments strongly implicate this compound as a significant free ecdysteroid in *Limulus*, although confirmation of structure requires mass spectral analysis.

The change in concentration of free 20-hydroxyecdysone during the molt cycle is in agreement with data from crustaceans[9], insects[10], and arachnids[11]. Although ecdysteroids may exist in conjugated form, as Bebbington et al.[12] suggest, it seems reasonable to conclude that the free ecdysteroid component, which occurs at ng/g levels and show increased concentrations during premolt, plays a major role in the molting physiology of *Limulus*.

Limulus larvae also exhibit the ability to rapidly convert ecdysone to 20-hydroxyecdysone as insects and crustaceans do[13]. Therefore it appears likely that the basic molting physiology and biochemistry of *Limulus* is similar to crustaceans, insects and probably all arthropods.

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3 A. Krishnakumaran and H. Schneiderman, Biol. Bull. *139*, 520 (1970).
4 T.C. Jegla, J.D. Costlow and J. Alspaugh, Gen. comp. Endocr. *19*, 159 (1972).
5 R.B. Gagosian, R.A. Bourbonniere, W.B. Smith, E.F. Couch, C. Blanton and W. Novak, Experientia *30*, 723 (1974).
6 P. Karlson and E. Shaaya, J. Insect Physiol. *10*, 797 (1964).
7 D.A. Schooley and K. Nakanishi, in: Modern Methods of Steroid Analysis, p.37. Ed. E. Heftmann. Academic Press, New York 1973.
8 T.C. Jegla and J.D. Costlow, Gen. comp. Endocr. *14*, 295 (1970).
9 A. Willig and R. Keller, J. comp. Physiol. *86*, 377 (1973).
10 D.W. Borst, W.E. Bollenbacher, J.D. O'Connor, D.S. King and J.W. Fristrom, Dev. Biol. *39*, 308 (1974).
11 J.C. Bonaric and M. DeReggi, Experientia *33*, 1664 (1977).
12 P.M. Bebbington, E.D. Morgan and C.F. Poole, in: Marine Natural Products Chemistry, p.367. Ed. D.J. Faulkner and W.H. Fenical. Plenum Press, New York 1977.
13 D.S. King and J.B. Siddall, Nature *221*, 955 (1969).

Estrogen action in the male[1]

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Summary. Estrogen administration induces a migration of eosinophil leukocytes to ductus deferens.

Estrogens are known to be synthetized in the mammalian testis[2–4]. Specific estrogen-binding proteins were identified in the male reproductive organs in several species[5–7], including man[8]. It was suggested that estrogens might be involved in modulatory mechanisms of testosterone synthesis[4], as well as in the regulation of its action in target organs[7]. However, the physiological role of estrogens in the male reproductive system still remains unclear.

In the mammalian female, estrogens induce migration of eosinophil leukocytes to the uterus[9], where they have been proposed to mediate some parameters of estrogen stimulation[10]. The aim of the present work is to investigate if similar mechanisms of estrogen action are also present in the male.

Material and methods. Mature male Sprague-Dawley rats were used in the present experiments. 60 µg estradiol 17 β/100 g b.wt in saline were injected s.c. and the animals were sacrificed 24 h after estrogen or vehicle injection. Testis, epididymis, prostate, ductus deferens and seminal

Estrogen-induced tissue eosinophilia in male sexual organs

Organ	Average number of tissue eosinophils/mm^2 ± SEM Control untreated rats	Rats 24 h after estrogen treatment	Variation in tissue eosinophils after estrogen treatment, expressed as Δ% over the controls
Testis	2.9 ± 0.7	1.3 ± 0.2	44.8 (n.s.)
Epididymis	5.7 ± 0.7	5.7 ± 1.2	100.0 (n.s.)
Prostate	8.3 ± 1.3	6.2 ± 0.9	74.6 (n.s.)
Ductus deferens			
Lamina propria	69.4 ± 7.8	263.5 ± 23.1	379.6 (*)
Muscular layer	15.2 ± 1.7	46.2 ± 4.4	303.9 (*)
Seminal vesicle			
Lamina propria	6.3 ± 0.8	10.7 ± 1.6	169.8 (n.s.)
Muscular layer	11.4 ± 1.4	17.0 ± 2.9	149.1 (n.s.)

* $p < 0.001$; n.s., not significant.