Research article

Ecdysteroids and oocyte development in the black fly *Simulium vittatum*

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Abstract

Background: Oocyte development was studied in the autogenous black fly, *Simulium vittatum* (Diptera, Nematocera), a vector of *Onchocerca volvulus*, the causative agent of onchocerciasis.

Results: Oocyte growth was nearly linear between adult eclosion and was complete by 72 hours at 21°C. The oocyte became opaque at 14 hours after eclosion indicating the initiation of protein yolk deposition. The accumulation of vitellogenin was measured using SDS-PAGE. The density of the yolk protein bands at about 200 and 65 kDa increased during the first and second days after eclosion. The amount of protein in the 200 kDa band of vitellogenin, determined using densitometry, rapidly increased between 12 and 25 hours after eclosion. Ecdysteroid levels were measured using a competitive ELISA. Ecdysteroid levels increased rapidly and subsequently declined during the first day after eclosion.

Conclusion: These data show a correlation between the appearance of vitellogenin in the oocyte, and the rise in ecdysteroids. A possible relationship to molting of the nematode, *Onchocerca volvulus*, is discussed.

Background

Blackflies are major nuisance pests and are vectors of the nematode, *Onchocerca volvulus*, which causes the serious human disease, onchocerciasis, mainly in tropical Africa but also in Central and South America. Microfilariae, ingested by the black fly from the human host, invade the thoracic muscles and molt several times. The steroid hormone, 20-hydroxyecdysone, is known to control molting in insects [1] and has been implicated in the control of molting of nematodes [2]. Ecdysteroids have been isolated from several parasitic nematodes [3], and have been shown to fluctuate in titer during the molt [4]. Genes related to the 20-hydroxyecdysone receptor gene of *Dro-*

sophila melanogaster have been isolated from *O. volvulus*[5]. Interestingly, 20-hydroxyecdysone caused premature timing of the third stage molt of *Dirofilaria immitis*[6]. This raises the possibility that changes in ecdysteroid titer in the insect host could affect the timing of developmental molts of the parasitic nematode.

20-hydroxyecdysone is important in the control of reproduction in Diptera, for example in *Drosophila melanogaster*[7,8], the stable fly, *Stomoxys calcitrans*[9] the blowfly *Phormia regina*[10], and the mosquitoes, *Aedes aegypti*[11,12], *Aedes atropalpus*[13], and *Anopheles albimanus*[14]. Ecdystroid titers rise and fall in these insects
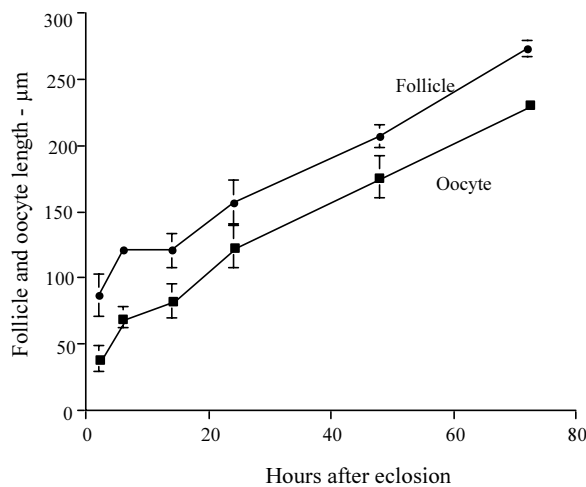
Figure 1
Follicle and oocyte length after eclosion in the black fly, *Simulium vittatum*. Each time point represents the mean of 20 follicle measurements from each of 3–5 females, ± standard deviation.

during the development of eggs. In the hemaotophagus Diptera, the titer of ecdystroids rise in the adult female after the blood meal as eggs develop [10,15,16]. It is possible that ecdysteroid titers also increase during egg development in the adult female black fly.

Simulium vittatum is one of the few blackflies that can be reared in the laboratory [17] making it especially useful for physiological studies. *S. vittatum* can develop its first batch of eggs autogenously [i.e. without a blood meal] but requires a blood meal for subsequent cycles of egg development [18]. We studied the relationship between egg development and ecdysteroids during autogenous egg development in this species.

Results
To determine the basic characteristics of oogenesis, black-flies were collected and follicle and oocyte lengths were measured. Oocyte growth in *S. vittatum* begins after adult eclosion and is completed by 72 hours after eclosion at 21°C. From 2–72 hours the increase in follicle and oocyte length was nearly linear (Fig. 1). The oocyte became opaque at 14 hours after eclosion indicating the initiation of protein yolk deposition.

To correlate the growth of the oocyte with the appearance of yolk proteins, the accumulation of protein in ovaries was measured using SDS-PAGE. As shown in Figure 2, major protein bands are present at about 200 and 65 kDa. These bands were not seen in extracts of males (data not shown). Given their presence in large amounts in the

oocytes, their absence in males, and the presence of similar bands in *Simulium ornatum* that have been immunologically identified as vitellogenin [23], we conclude that they represent the vitellogenin proteins of *S. vittatum*. The density of the vitellogenin bands increased during the first day after eclosion. The amount of protein in the 200 kDa band, determined using densitometry, increased linearly for 36 hours after eclosion (Fig. 3).

Ecdysteroid levels were measured using a competitive ELISA. Ecdysteroid levels increased rapidly and subsequently declined during the first day after eclosion (Fig. 4).
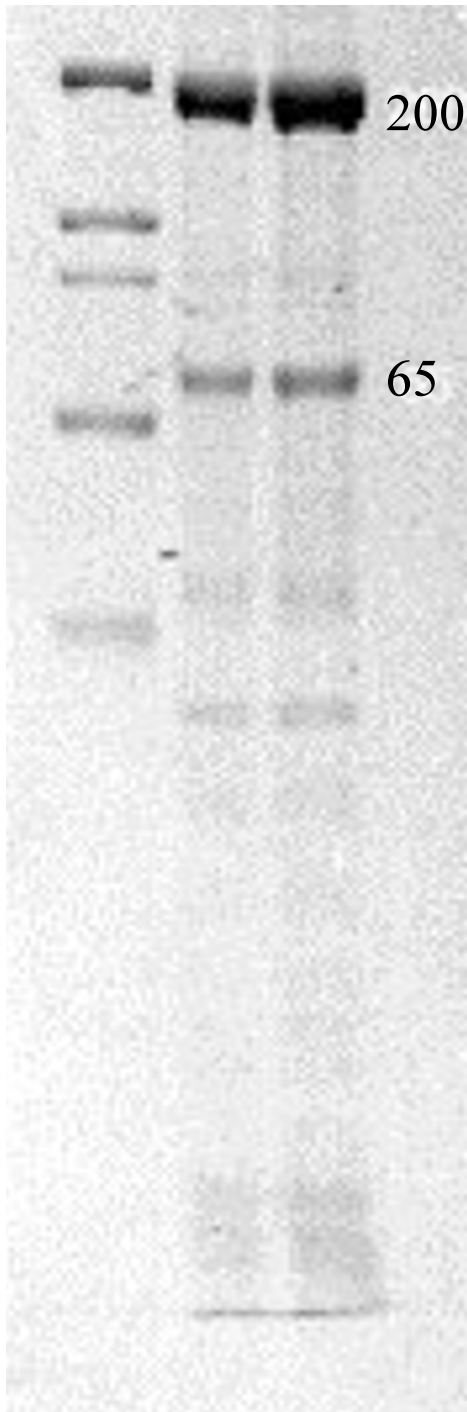
Discussion
These experiments demonstrate a correlation between growth of the oocyte, the appearance of vitellogenin in the ovary and rising titers of ecdysteroids in the black fly *S. vittatum*.

Previtellogenic growth of the follicle in *S. vittatum* takes about 12 hr. Ovarian follicles were observed to enter the vitellogenic stage at 14 hr after eclosion as indicated by the opaque appearance of the oocyte that occurs as yolk proteins are taken up. These observations correlate with those of Liu and Davies, [24]. Follicle growth was linear after eclosion. The oocyte reached maximum size by 72 hr after eclosion.
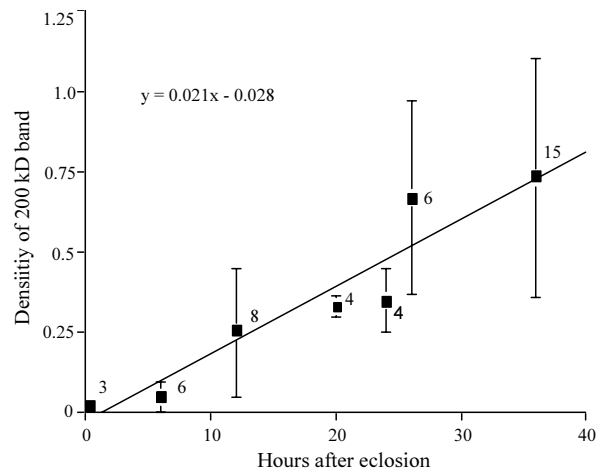
The appearance of opaque oocytes after 14 hr was correlated with an increase in the amount of vitellogenin present in oocytes.

Ecdysteroid levels were found to increase during the first day after eclosion. The changing titers of ecdysteroids were correlated with the appearance of vitellogenin in the oocytes and to the vitellogenic phase of oocyte growth. Vitellogenic growth of oocytes and vitellogenin synthesis are known to be stimulated by ecdysteroids in several cyclorrhaphid flies and mosquitoes [7–10,12]. This suggests that egg development in *S. vittatum* may also be regulated by ecdysteroids. Confirmation that ecdysteroids regulate vitellogenin synthesis in *S. vittatum* would require more direct evidence.

These observations are similar to those of the autogenous mosquito, *Ae. atropalpus*[14]. The timing of the growth of the follicle, and the rise in vitellogenin and ecdysteroids are similar in both species. In contrast, vitellogenic growth does not occur until after a blood meal in the anautogenous black fly, *Simulium ochraceum*[25], or the anautogenous mosquito *Ae. aegypti*[26]. In *Ae. aegypti* the vitellogenic period, including the rise in ecdysteroids and the synthesis of vitellogenin, does not begin until after the blood meal. Examining ecdysteroid titers after a blood

$y = 0.021x - 0.028$

Figure 3
Vitellogenin accumulation in *Simulium vittatum* ovaries after eclosion. Ovaries were homogenized in SDS sample buffer. Proteins were separated on SDS-PAGE and stained with Coomassie blue. The intensity of the stain in the 200 kDa protein band was determined by densitometry. Data are reported as the mean ± standard deviation relative to the density of the 200 kDa band at 12 hours to correct for differences in staining intensity between gels. The number of band measurements used to calculate the mean is shown next to the data points.

Figure 2
SDS-PAGE Separation of vitellogenin protein in *Simulium vittatum* on SDS gels. Lane 1, molecular weight markers (200, 116, 97, 66 and 45 kDa). Lane 2, ovaries with proteinase inhibitors; lane 3, ovary extract without proteinase inhibitors. Ovaries were removed 36 hours after eclosion.
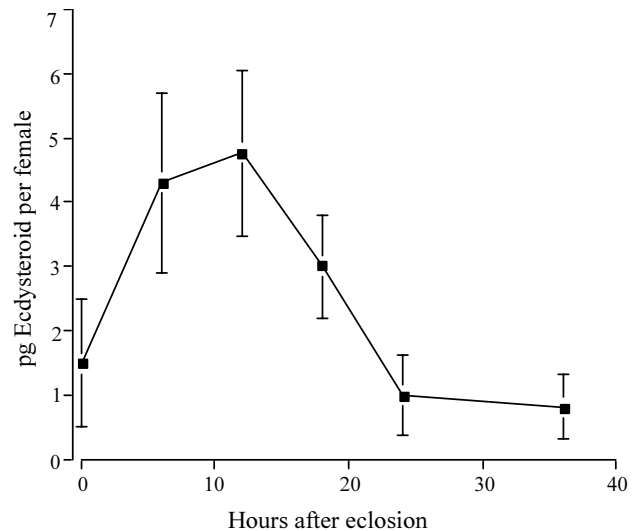
Figure 4
Ecdysteroid levels in *Simulium vittatum* with time after eclosion. Ecdysteroid levels were measured using a competitive ELISA as described by Kingan (21). Each point represents the average of 9 groups of 10 females, ± standard deviation.

meal in an anautogenous black fly species would be of interest.

Conclusions

Our data suggest that microfilariae of *O. volvulus* would be exposed to changing titers of ecdysteroids during their development within the thoracic muscles. Development of microfilariae to the infective third stage takes 11 days in *Simulium ochraceum* during which time the host could complete 3 gonotrophic cycles [27], and 6 to 8 days in *Simulium damnosum*[28]. Given that the complete development of eggs in *S. vittatum* takes 3–4 days [29], the microfilariae might be exposed to several peaks of ecdysteroids during their development. It is possible that development of microfilariae is cued by the fluctuating titers of ecdysteroids in the host.

Methods

Blackflies, *Simulium vittatum*, were reared using the method described by Cupp and Ramberg [19]. Females were collected within 1 hr intervals under simulated natural conditions and stored in an incubator at 21°C. The adult females were fed 15% dextrose containing 1% streptomycin and mycostatin.

Follicle growth measurement

Ovaries were removed in *Aedes* saline [20] and follicles were teased apart using minuten needles. The follicle of the black fly is polytrophic and therefore contains an oocyte and accompanying nurse cells. Follicle and oocyte lengths were measured at different times after eclosion using a compound microscope and an ocular micrometer.

Ecdysteroid ELISA

Females were collected at various time points after adult eclosion, frozen, and homogenized in 50% MeOH (10 females / 600 ml). The homogenate was centrifuged and the supernatant was stored at -20°C. Ecdysteroid titers were assayed using a competitive ELISA as described by Kingan [21] using 20-hydroxyecdysone as the standard. The assay is about equally sensitive to ecdysone and 20-hydroxyecdysone. The latter is considered to be the active form of the hormone. The primary anti-ecdysone antibody was a generous gift of T.M. Kingan, University of California at Riverside. The secondary antibody was anti-rabbit IgG alkaline phosphatase conjugate. Activity was detected using p-nitrophenyl phosphatase [pNPP, Immunopure tablet from Pierce]. The absorbance was measured using an ELISA plate reader at 405 nm. Data analysis was performed as described by Kingan [21] using "Softmax" data analysis program (Molecular Devices Corp., Palo Alto, CA).

SDS–polyacrylamide gel electrophoresis

Females to be assayed were dissected in *Aedes* saline. Ovaries from two females were removed and stored at -70°C.

Ovaries were disrupted in 50 µl of sample buffer (5 mM Tris Base, 4% SDS, 0.002% Bromophenol Blue, 20% glycerol, pH 6.8, 2% mercaptoethanol) for 90 seconds in a microwave oven on high setting and then placed in boiling water for 1 minute at 100°C as described by Horscroft and Roy [22]. The mixture was then centrifuged in a microfuge for 3 minutes. Including protease inhibitors (aprotinin, chymostatin, antipain, leupeptin, all at 5 µg/ml, plus 1 mM PMSF and 5 mM benzamidine) had no effect on results presumably because boiling in SDS rapidly inhibited proteases (see Fig. 2). Proteins in the supernatant (5–20 µl) were separated electrophoretically using a vertical slab unit (Hoefer Scientific Instruments) on 8.25% SDS-polyacrylamide gels. The molecular weight standard (Biorad, high molecular weight) had markers at 200, 116, 97, 66 and 45 kDa. Gels were stained with Coomassie Brilliant Blue and analyzed using a densitometer (Ultrascan XL Enhanced Laser Densitometer, Bromma). The density of the 65 kDa and 200 kDa vitellogenin bands were measured.

Abbreviations used

ELISA: enzyme linked immunosorbant assay

Competing interests

None declared

Acknowledgements

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