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Hormonal modulation of cannibalistic behaviors in mosquito (*Culex pipiens*) larvae

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ABSTRACT

Cannibalism has been observed occasionally in a limited number of different animal species, but the underlying mechanisms that foster this behavior are mostly unknown. Here we show that mosquito (*Culex pipiens*) larvae show this behavior towards conspecifics under certain conditions. Cannibalism was only observed in 4th instar larvae and only in response to starvation. Well fed animals never showed any cannibalistic behavior. Starvation induced cannibalism of *Culex* 4th instar was predominantly directed towards 3rd instars rather than to 1st or 2nd instar larvae. Specific mandibular structures of these larvae enable this cannibalistic behavior. We could show that treatment with the biogenic amine octopamine, which is known to be involved in the control of starvation and aggression, increased the rate of cannibalism of food-deprived 4th instar larvae significantly. Incubation with the octopamine receptor antagonist phentolamine suppressed this cannibalistic behavior. Moreover, octopamine not only increased the rate of cannibalism, it also induced a shift towards smaller prey. A role of octopamine in this starvation induced behavior was further supported by direct measurements of the total content of this important neuroactive compound. Taken together, we could show that 4th instar mosquito larvae showed cannibalistic behavior after starvation and that this behavior apparently depends on octopamine.

1. Introduction

Cannibalism, the killing and eating of conspecifics (14; 21) was considered to be an uncommon and incidental phenomenon in animals with only little evolutionary significance (Costa and Pérez-Miles, 2002; Evans, 1998). Reliable reports of cannibalistic behaviors have been published for a very limited number of animal species. Most often, cannibalism was observed among immature stages of a variety of insect species such as the bark beetles *Scolytus multistriatus* (Marsham), *S. scolytus* (F.), and *Tomicus piniperda* L., where large larvae consume smaller conspecifics they encounter while boring in phloem (Beaver, 1974), the moth *Carmenta hematica* (Ureta) (Clark and Jackson, 1994), and the chrysomelid beetle *Labidomera clivicollis* (Kirby) (Denno et al., 2002). Caterpillars of the Indian meal moth, *Plodia interpunctella*, can consume even larger conspecifics, if they are weakened by a granulosis virus (Bilde and Toft, 2001). Very specific cases of cannibalism have been reported for the blood sucking kissing bug *Rhodnius prolixus*, where young larvae feed on older ones that took a large blood meal (Lazzari et al., 2018). Cannibalistic behaviors may enhance the fitness of the cannibal as conspecifics are used as a valuable food source and the number of potential competitors is reduced. However, it can also have negative effects by increasing the spread of pathogens and parasites, if cannibalized individuals are infected. Different studies employing divergent models have been performed. Actually, the occurrence of cannibalism upon starvation and also in the presence of sufficient food has been reported in *Toxorhynchites* and *Anopheles* mosquitoes (Koenraadt et al., 2004; Porretta et al., 2016; Rajavel, 1992; Scharf, 2016). But mechanisms that might assist the transition from a normal towards a cannibalistic lifestyle are not understood at all.

Starvation and aggressiveness are major factors that are believed to be relevant for this transition towards a cannibalistic behavior. Periods of starvation may increase the willingness of individuals to eat conspecifics and enhanced aggressiveness is obviously required to kill them. Physiological adaptation that are needed to cope with starvation are known to be mediated through release of specific neurohormones, with octopamine (2-amino-1-(4-hydroxyphenyl)ethanol) being the most important one in invertebrates (Li et al., 2016; Li et al., 2017; Roeder, 1999, 2005). Moreover, aggressive behaviors are also controlled by neurohormones, in this case also by octopamine and dopamine (3,4-dihydroxyphenethylamine). Both biogenic amines act as

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neurotransmitters, neurohormones and neuromodulators. They are responsible for regulating many biological processes including locomotion, learning and memory (Riemensperger et al., 2005; Schwaerzel et al., 2003; Unoki et al., 2005) and aggression (Roeder, 2005; Roeder et al., 2003; Zhou et al., 2008). Despite their potential relevance for controlling cannibalistic behaviors, our knowledge about their actual role for this interesting behavior is very limited.

Thus, we wanted to elucidate if 4th instar larvae of *Culex pipiens* show cannibalistic behavior against younger vulnerable conspecific larvae and if this behavior depends on the nutritional state of the potential cannibal. Moreover, we wanted to know if the neurohormone octopamine and its antagonists are involved in this cannibalistic behavior of larval *Culex pipiens* individuals.

2. Material and methods

2.1. Mosquito rearing

Mosquito egg rafts were collected from a breeding site in the Quhafa region, Tanta City, Egypt. Hatched larvae were identified as *Culex pipiens* according to Harbach (1988). They were placed in white enamel pans containing dechlorinated tap water. Larvae were reared at insectary conditions of 27 ± 2 °C, 70–80% relative humidity and under a 12:12 h (light: dark) photoperiod. The larvae were fed Tropical Fish Food (Tetra GmbH, Melle, Germany).

2.2. Lip3 expression level

To identify physiological signs of starvation, we used *lipase 3* (*lip3*) expression levels as a marker (Fuss et al., 2006). Thus, we determined lip3 transcript levels by semi-quantitative RT-PCR. Briefly, three groups each of ten 4th instar larvae were fed on 0.1, 0.176 or 0.23 mg food mixture (1 yeast: 2 grinded rusks)/100 ml of culture water. The same number of 24 h food-deprived larvae was used as control. Total RNA was extracted from 4th instar larvae using the GeneJET RNA purification kit (Thermo Scientific, Waltham, USA) and converted to first strand cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, USA) according to the manufacturer's protocols. The reverse transcription product was used directly as a template in PCR amplification. Primers and condition of RT PCR analysis for Lip3 gene was described previously (Wang et al., 2015). β-Actin was used as a house-keeping gene. Experiments were performed in triplicate. PCR products were loaded in 1% agarose gel supplemented with Ethidium Bromide. The band intensity as an indicator of relative expression of *lipase 3* was measured using ImageJ software (http://rsb.info.nih.gov/ij/index.html). Numerical data obtained from each experiment were presented as mean ± standard deviation. Means were compared using one-way ANOVA. The level of significance was set at $p < 0.05$.

2.3. Effect of starvation on cannibalism

Fourth instar *Culex pipiens* larvae were food-deprived for 24 h before the experiment. Cannibalism of food-deprived 4th instars against 1st, 2nd and 3rd instar was evaluated in separated populations. Well-fed larvae were used as controls in this experiment. During the experiment, containers were placed on lab bench and cannibalism was evaluated by counting and determining the instar of cannibalized larvae at time intervals of 24 h, 48 h and 72 h. All cannibalism assays were done in triplicate.

2.4. Effect of octopamine and its antagonist on cannibalism

This experiment was designed as described above, except that the 24 h food-deprived mosquito larvae were exposed to dechlorinated water supplemented with 10^{-4}M octopamine or 10 mM phentolamine. Food-deprived larvae in normal dechlorinated water without octopamine and its antagonists were used as control. A series of experiments was designed to determine these optimal doses, which induce changes in behavior with minimal compound concentrations (data not shown). Data was recorded as mentioned above.

2.5. Light microscopy

The remaining parts of cannibalized larvae, dissected guts of cannibal and control 4th instar larvae were transferred to glass slides using a fine brush for microscopic examination using an Olympus BX61 light microscope at a magnification of 2.5 × 10.

2.6. Scanning electron microscopy

SEM was done in order to focus on the mouthparts which enable *Culex pipiens* larvae to cannibalize each other. Briefly, heads of 4th larval instar of *Culex pipiens* were fixed in 2.5% buffered glutaraldehyde in 0.1 M PBS, PH 7.4 at 4 °C for 2 h, and washed three times in PBS (10 min each). Samples were then fixed in 1% Osmic acid (30 min) and washed three times in PBS (10 min each). Then, samples were dehydrated in a series of ethanol (30, 50, 70, 90 and 100%) infiltrated with acetone, each concentration for 30 min. Samples were dried using liquid CO_2 and mounted in a straight position with mouth parts looking up on aluminum stubs, coated with gold in a SPI-Module™ Vac/Sputter 7. Photographs were captured using JEOL, JSM- 52500 LV scanning electron microscopy (JEOL; JSM-5200 LV, Tokyo, Japan) at 20 KV.

2.7. Measurement of octopamine

Total octopamine concentration in normal, food-deprived and cannibalistic 4th instar *Culex pipiens* larvae were measured using high-performance liquid chromatography (HPLC). Briefly, samples were grinded in a mixture of acetonitrile and methanol (1/1) followed by sonication (Ultrasonic, Frequency 75 kHz) for 15 min. The samples were centrifuged for 5 min at 5000 rpm. The supernatants were allowed to dry in order to concentrate the samples. Quantitiative HPLC was carried out using system equipped with a binary pump (LC 1110; GBC scientific Equipment, Hampshire, USA) and C18 column (Kromasil C18, 5 µm, 150 × 6.4 mm Sigma Aldrich, St Louis, USA;), with an injection volume of 20 µl, with isocratic procedure where the mobile phase was methanol-acetonitrile-sodium pentane salphonate (7.5:7.5:85, v/v/v) and the flow rate 0.85 ml/min. The ultraviolet detector (LC 1200; GBC Scientific Equipment, Hampshire, USA) with the wavelength of 275 nm was used to detect and quantitate octopamine where individual standard curves were prepared using different concentrations of standard used for calculation of each sample.

2.8. Statistical analysis

Data obtained from cannibalism assays were presented as means ± SD. The results of cannibalism of control versus starved larvae and starved versus OA and OA treated larvae were compared using Student's *t*-test, for data in Figs. 3 and 5 with Bonferroni correction. Data obtained from HPLC were quantified and analyzed using Win Chrome Chromatography Ver. 1.3 software.

3. Results

In order to study cannibalistic behavior and its underlying mechanisms, we used culicine mosquito larvae as a model. The feeding behavior of these larvae kept under laboratory conditions consists of foraging along the bottom of the container, and stationary filter feeding from the water surface film. In this study, we confronted single food-deprived (24 h) 4th instar larvae with 3 individuals of each instar. For the next three days, the experiments were recorded. Cannibalism could be observed repeatedly and the cannibalistic 4th instar larvae ingested