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Role of Catecholamines in the Reproduction of *Romanomermis culicivorax*[1]

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Abstract: The relative concentrations of catecholamine in the nervous system of the entomophilic nematode *Romanomermis culicivorax* were measured under different experimental conditions by a glyoxylic acid-induced fluorescence procedure. A greater concentration of catecholamine was recorded in the nervous system of adult males and females than in postparasitic juveniles. A higher concentration of catecholamine occurred in adults maintained in physical contact with the opposite sex than in those maintained in isolation. Adult males maintained with females in the same aqueous medium but physically separated by a barrier displayed a greater concentration of catecholamine in their nervous systems than did males maintained in isolation, but the catecholamine fluorescence intensity of such males was less than in males allowed physical contact with females. In adult males, the fluorescence intensity of catecholamine declined progressively during and after copulation. In adult females, the intensity of catecholamine remained constant before, during, and after copulation. Catecholamine(s) may play a role in regulating copulatory behavior, egg production, or oviposition.

Key words: catecholamine, fluorescence, glyoxylic acid, nematode, nervous system, reproduction, *Romanomermis culicivorax*.

The role of catecholamines as neurotransmitters has been documented in a variety of animal phyla (23,24). Among nematodes, suggested roles for aminergic neurons include mechanoreception in *Caenorhabditis elegans* (22), *Prionchulus punctatus* (25) and *Panagrellus redivivus* (20), chemoreception in sensilla of *Trichinella spiralis* (17), and coordination of copulatory behavior in *Goodeyus ulmi* (16), *P. redivivus, Nematospiroides dubius* (20), and *Nematodirus battus* (21). In addition, catecholamines, especially noradrenaline, may control molting in *Haemonchus contortus* (5,19) and *Phocanema decipiens* (7–9).

Romanomermis culicivorax is a mermithid nematode parasite in the hemocoel of larval mosquitoes. After exiting the host, postparasitic juveniles molt to adults, which mate and oviposit within the substratum of the larval host's environment. Only the parasitic stages feed.

Recently, we demonstrated that *R. culicivorax* possesses a well-organized system of catecholamine-producing neurons and that this system develops in an incremental fashion as the nematode progresses through parasitic and postparasitic development (14). The presence of catecholamines in the amphids and cephalic papillary nerves constitutes circumstantial evidence for the role of such compounds in mediating sensory information. Additionally, the fact that adult male nematodes possess 16–20 catecholaminergic ganglia in the tail region, whereas females possess only two such ganglia, suggests that catecholaminergic centers may be involved in regulating copulatory behavior and, possibly, other aspects of reproduction. Therefore, the purpose of the present investigation was to examine the possibility that catecholamines may be involved in controlling reproduction in *R. culicivorax*.

Materials and Methods

Experimental design: The relative concentrations of catecholamine in the nervous system of *R. culicivorax* were compared in the following organisms: (a) males only, (b) females only, (c) males and females incubated together, and (d) males and females separated physically but connected via the same maintenance medium, sterile water.

The laboratory colony of *R. culicivorax*

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was maintained in vivo with the general technique of Platzer and Stirling (18), except that the host was the mosquito *Aedes aegypti* instead of *Culex pipiens*. Also, postparasitic stages were maintained in sterile water, because we have found that the local water supply carries a bacterium pathogenic to the nematodes. Immediately after emergence from the host, postparasitic nematodes were collected in a petri dish containing sterile water, sexed, distributed among four sets of petri dishes according to conditions (a) to (d), and maintained in an incubator at 27 C. Under these conditions, molting to the adult stage occurred ca. 5 days after emergence from the host in all regimes, whereas mating occurred within 24 hours afterward in regime (c).

Each experimental unit consisted of a covered glass petri dish (9.0-cm-d) containing sterile water. The base of a 5.5-cm-d plastic petri dish was placed upright inside the larger dish; the sides of the smaller petri dish were perforated with small holes. Thus, nematodes placed inside the smaller petri dish were physically separated from those placed in the larger dish, but chemical communication between the two groups of nematodes could occur via the water that connected them. In treatments (a) and (b), 100 males or females were transferred into each perforated dish; in (c), 100 males and 100 females were transferred into each perforated dish; in (d), 100 males were transferred into each glass petri dish and 100 females into each perforated petri dish. Each petri dish system was considered as a separate replicate, and there were four replicates per treatment.

In all treatments, the relative concentration of catecholamine in the nerve rings of males and females was measured 3 days after emergence from the host and immediately after the last molt. The catecholamine in the nerve ring of males and females incubated together (c) was also measured during the first or second day of mating (a process that lasted ca. 5–6 days) and within 24 hours after mating, i.e., when the sexes had separated from one another. Nematodes that were not mating were removed from the main cluster of nematodes within 24 hours after the last molt. The remaining nematodes provided specimens for catecholamine determinations during and after mating. The concentration of catecholamine in tail ganglia was measured for adult male nematodes only, because the tail of adult females contained only two ganglia that stained inconsistently, whereas the tail of adult males contained many ganglia that stained consistently for catecholamine; staining of the ganglia did not occur before the last molt (14). Catecholamine fluorescence intensity in male tail region ganglia was determined immediately after the last molt in all treatments and during and after mating for treatment (c). Fifteen nematodes were selected for the measurement of catecholamine fluorescence intensity from each replicate at all developmental stages examined.

Relative catecholamine concentrations: Catecholamine concentration within neurons was measured by modifying a glyoxylic acid (GA)-induced fluorescence method, developed for nematodes (20), that differentiates catecholamines from other amines (6). A solution of 2% (w/v) GA in 0.1 M Na_2HPO_4–KH_2PO_4 buffer (pH 7.0) was used. Best results were obtained when nematodes were killed quickly before transfer to GA, as live nematodes put into cold GA usually shrank and became distorted. Killing of the nematodes was achieved by placing them in a 100-ml beaker containing ca. 50 ml water, which was then plunged into another beaker filled with boiling water for 2–3 minutes or until the specimens assumed the almost straight form characteristic of heat death (12). The killed nematodes were incubated at room temperature (24–27 C) in a beaker containing 2% GA for 5 minutes and transferred in small drops of GA onto slides. The excess GA was removed immediately with filter paper and the slides were dried rapidly (to reduce background fluores-

cence) with a stream of hot air from a hair-dryer for 5 minutes. The slides were then heated at 100 C in an oven for 10 minutes. Nematodes were mounted in Histoclad (Clay Adams, Parsippany, NJ) under a coverslip and examined immediately.

The fluorescence intensity (%) of catecholamine was determined by the method of Björklund et al. (1), with a synthetic catecholamine standard. The standard consisted of 2.0 mM dopamine (3-hydroxytyramine; Sigma Chemical Co. St. Louis, MO) plus 5% (w/v) human serum albumin (Sigma) in 0.1 M phosphate buffer (pH 7.0). With a fine-tipped pasteur pipet, a small drop of this solution was placed on a glass microscope slide, dried at room temperature, treated with 2% (w/v) GA for 5 minutes, and then dried, heated, and mounted in Histoclad as with biological material. The same size droplet (23.7 ± 0.7 µl; $n = 20$) was used as a dopamine reference standard in all experiments; mean dopamine content of the droplet was 4.74×10^{-2} µmoles.

Fluorescence microscopy: Specimens were viewed with a Zeiss Photomicroscope II, equipped with a 50 W mercury UV epifluorescence lamp and a Plan-Neofluar 25/0,8 Immersion objective. A wide-band interference filter 18 (excitation band pass 390–440 nm; barrier filter long pass 470 nm) was used.

The fluorescence intensity of catecholamine in the nervous system was measured with an MPM3 photometer head and SF14 digital voltmeter controlled by the shutter switching system connected to the photomicroscope. The measuring field was restricted by a series of interchangeable pinhole diaphragms (apertures) in the photometer head. For the measurement of percent fluorescence intensity of catecholamine in the four ganglia of the nerve ring of adult nematodes, a 0.32-mm-d pinhole aperture was used, whereas the pinhole aperture used for the tail of males was 0.16-mm-d, because the tail ganglia were smaller than those in the nerve ring. The digital voltmeter was calibrated to 100 with respect to the fluorescence intensity of the synthetic dopamine standard. Values obtained for the nematodes were expressed as a percentage of the standard.

Statistical analysis: To preserve normality and homogeneity of variance in the data, an arcsine transformation was applied. ANOVA was used to determine the significance of mean fluorescence intensities within and between the rearing conditions. The conditions were compared using Duncan's multiple-range test.

Results

There were no significant differences between male and female postparasitic juveniles in the mean fluorescence intensity of catecholamine in their nerve rings (Table 1). The fluorescence intensity was unaffected by the physical or chemically mediated presence of the opposite sex. The concentrations of catecholamine in the nerve rings of adult males and females (Table 2) was greater than in the nerve rings of postparasitic juveniles (Table 1), when comparable sexes and experimental groups were compared.

The catecholamine fluorescence intensity was higher in adult males and females incubated together than in adults kept in complete isolation of the opposite sex (Table 2). The catecholamine concentration in

Table 1. Effect of presence of the opposite sex on the concentration of catecholamine in the nerve ring of postparasitic juvenile *Romanomermis culicivorax*.

	Catecholamine concentration	
Incubated nematodes	Male	Female
Males only	14.50 ± 0.48	
Females only		13.74 ± 0.54
Males and females separated	13.90 ± 0.56	13.55 ± 0.36
Males and females together	14.18 ± 0.52	13.72 ± 0.46

Values are expressed as percent intensity of the 4.74×10^{-2} µmole dopamine standard and are the means ± SE of four replicates of 15 nematodes. Means in each vertical column and horizontal row are not significantly different ($P > 0.05$) by Duncan's multiple-range test.

TABLE 2. Effect of presence of the opposite sex on the concentration of catecholamine in the nerve ring and tail ganglia of adult males and in the nerve ring of adult females of *Romanomermis culicivorax*.

	Catecholamine concentration		
	Male		Female
Incubated nematodes	Tail	Nerve ring	Nerve ring
		Before copulation	
Males only	3.62 ± 0.11 C	20.86 ± 0.65 z	
Females only			22.98 ± 0.34 zv
Males and females separated	4.05 ± 0.08 B	27.48 ± 0.65 y	24.01 ± 0.79 v
Males and females together	5.87 ± 0.12 A	34.73 ± 0.94 w	31.13 ± 1.16 x
		During copulation	
Males and females together	3.30 ± 0.08 D	22.12 ± 0.55 zv	27.67 ± 0.73 y
		After copulation	
Males and females together	3.22 ± 0.10 D	14.93 ± 0.83 u	31.76 ± 1.06 x

Values are expressed as percent intensity of the 4.74×10^{-2} μmole dopamine standard and are the means ± SE of four replicates of 15 nematodes. Means with the same lower case letter (down and across the columns) and means with same uppercase letter (down the single column) are not significantly different ($P > 0.05$) by Duncan's multiple-range test.

the nerve ring of adult males maintained in physical contact with females was almost double that in males maintained alone. Similarly, the catecholamine concentration in the tail ganglia of adult males maintained in physical contact with females was 50% higher than in males maintained in isolation of the opposite sex. Even when the males and females were separated by a barrier but in the same aqueous medium, the catecholamine fluorescence intensity in the nerve ring and tail ganglia was significantly greater in these males than in males maintained alone, although less than in males incubated physically with females.

The catecholamine fluorescence intensity in the nerve ring of adult females maintained in physical contact with males was ca. 50% higher than in females maintained alone or separated from males by a barrier in the same aqueous medium. Unlike males, females did not contain higher concentrations of catecholamine in their nerve rings than "females only" when chemical communication between the sexes via the aqueous medium was allowed.

The concentration of catecholamine in the nerve ring and tail ganglia of adult male nematodes declined progressively during and after copulation; the catecholamine concentration in the nerve ring of males after copulation was the same as in postparasitic juveniles (Tables 1, 2). In contrast, the concentration of catecholamine in the nerve ring of adult females remained approximately double that of postparasitic juveniles, both during and after copulation.

DISCUSSION

The studies showed that the physical presence of the opposite sex stimulated the production of catecholamine in the nerve ring ganglia of both sexes and in the tail ganglia of the adult males. In males, this increased catecholamine production partially resulted, in the absence of physical contact with females, from chemical mediation between the sexes through the medium in which the nematodes were maintained. This result suggests that the catecholamine is produced as a facilitator or as a consequence of copulatory behavior. The possibility that catecholamine may play a role in sex attraction deserves further investigation, especially because sex attractants of an undetermined nature are secreted by female *R. culicivorax* (W. Hominick, pers. comm.). The production of sex attractants by female nematodes has been documented for a variety of nematode species (2,10,11).

The presence of catecholaminergic ganglia in the tail of adult males and their con-

nection with the caudal sensory papillae (14) suggest that the catecholamine may function, at least in part, to regulate copulatory behavior. The observation that the concentration of catecholamine declined progressively in the males during and after copulation suggests that the role of catecholamine in males may be limited to sex attraction and the initial stages of copulatory behavior. Because the fluorescence intensity of catecholamine in females both during and after mating remained approximately double that of postparasitic juveniles, catecholamine could be involved in regulating egg formation or oviposition. This suggestion is consistent with the fact that exogenous application of biogenic amines alters egg laying in *C. elegans* (3,13).

Although neurosecretions of various chemical types have been reported from a variety of nematode species (4), their roles in regulating developmental and physiological processes are poorly understood. *Romanomermis culicivorax* possesses at least two distinct neuronal systems: one secretes a peptide with immunocytochemical properties analogous to the molluscan hormone FMRFamide (15); the other produces catecholamines (14). The present study indicates that reproductive development is correlated with catecholamine production. Further studies are needed to more fully determine the role of the nervous system in regulating specific development events.

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