Chromatographic Fractionation of Aggregation and Sex Pheromones of *Nippostrongylus brasiliensis* (Nematoda)

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ABSTRACT Gel filtration with Sephadex G-25 or Bio-Gel P-2 of homogenates of the sexes of the zooparasitic nematode *Nippostrongylus brasiliensis* revealed two fractions with pheromonal activity. One active fraction with a suggested molecular weight over 500 was found in aqueous extracts of both helminth sexes and was attractive to both sexes by in vitro bioassay. A second region of pheromone activity was found in only female *Nippostrongylus* and was attractive to only male helminths. A molecular weight under 400 is proposed for this region. Additionally, a third region that represented ionic weights was present in both sexes, but attracted only female worms. These results indicated that helminth to helminth attraction is present at both species and sexual levels of recognition.

The sex pheromones of few nematodes have been examined chemically, and none have been identified. However, Stringfellow ('74) reported the hydroxyl ion as an attractant pheromone for *Pelodera strongyloides,* but recognized the possibility of additional pheromone compounds in this species. Green ('66) found that female *Heterodera schachtii* produced a less labile or more concentrated sex pheromone than did female *H. rostochiensis.* The female *Heterodera* pheromones were stable to ultraviolet light, drying, and moderate heat. Green ('66) reported that the pheromone exhibited little attractiveness after 24 hours at 20°C, while Greet et al. ('68) stated the *Heterodera* pheromone included volatile as well as non-volatile fractions. In contrast, Green ('67) and Cheng and Samoiloff ('71) reported that the female sex pheromones of *Panagrolaimus rigidus* and *Panagrellus silusiae,* respectively, were non-volatile.

Green and Plumb ('70) examined the female sex pheromones of ten *Heterodera* spp. and postulated, based on attractiveness to males of the various species, that at least six distinct attractive compounds were present. These pheromones were stable after drying, when stored at 5°C for one month in sealed containers.

Balakanich and Samoiloff ('74) utilized ether : water extraction to demonstrate that the female sex pheromone of *Panagrellus* strains was water-soluble. Treatment of the pheromone with Pronase decreased, but did not eliminate, the activity (Balakanich and Samoiloff, '74). Gel filtration chromatography of the pheromone revealed both higher and lower molecular weight components that exhibited strain-specific attractive and retentive properties (Balankanich and Samoiloff, '74).

Chemical characterizations of zooparasitic nematode pheromones are conspicuously absent, since only two reports have concerned this group. Anya ('76) found the female sex pheromone of *Aspicularis tetraptera* was more heat labile than the male-produced sex pheromone. Roberts and Thorson ('77) reported that *Nippostrongylus brasiliensis* females were attracted to male- or female-produced substances which moved with cholesterol and β-sitosterol on thin-layer chromatography.

This report presents our preliminary efforts in chemical research on the various pheromone systems of *Nippostrongylus brasiliensis.* Adequate chemical studies involved with pheromone isolation, characterization, and identification would greatly facilitate future exploration of nematode pheromone biology and provide clear-cut evidence of the chemical

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bases of nematode pheromonal attraction, thus surpassing the present, largely natural-history realm of worm interaction.

MATERIALS AND METHODS

Laboratory maintenance of mouse-adapted *Nippostrongylus brasiliensis* was conducted as previously reported (Bone et al., '77). Additionally, bioassay procedures were similar to those described previously (Bone et al., '78). All pheromone-source and bioassay nematodes were taken at five or six days post-infection.

Determination of the pheromone diffusion period for bioassay activity was studied with a 400 female-hour pheromone source (equals amount of pheromone produced by 400 worms in 1 hour) derived from an incubate prepared from the maintenance of the female nematodes in Tyrode's solution. Single male responders were placed in the center of the response portion of the bioassay chamber at zero or two hours after introduction of the pheromone into the source portion of the chamber. Mean distance traveled by the males was recorded at 30-minute intervals until four hours after addition of the pheromone. Forty replicates were performed for each diffusion period.

Lability of the female pheromone was investigated by storage of female incubate at −20, 1, 4, 22, or 37°C for 24 hours prior to bioassay of 400 female-hour samples. Additionally, sterile incubate solutions from membrane filtration (0.2 μm porosity) were tested similarly after 24 hours at 4, 22, or 37°C. Results were compared to a standard dose-response line reported earlier by Bone et al. ('78) for calculation of the relative specific activity (equals percentage of male locomotor response to fresh pheromome). Forty replicates were done for each treatment.

Chromatography was conducted on Sephadex G-25-40 (Pharmacia) in a glass column (0.7 × 27 cm) with glucose-free Tyrode's solution as an elutant. All procedures were performed at 4°C. Female worms were macerated manually in a tissue grinder with Tyrode's solution and centrifuged at 4,000 rpm for ten minutes prior to application of a 150 μl supernatant sample from 800-2,400 worm-equivalents (1 worm-equivalent equals amount of pheromone extracted from 1 helminth) to the column. Aliquots (600 μl) of the inclusion volume, using Blue Dextran and para-nitrophenol as standards, were collected and pooled by threes unless otherwise noted prior to bioassay. The heme fraction from helminths served as a convenient internal standard. Blue Dextran (exclusion volume) eluted in fraction 8.5 on Sephadex and fraction 8 on Bio-Gel while paranitrophenol peaked in fractions 32 and 22 on Sephadex and Bio-Gel, respectively. After localization of the active pooled samples by dosage-response bioassay, the activity of the individual fractions within each active sample was found by bioassay at a 60 female-equivalent dosage. Pooled samples from male helminths were handled similarly. Twenty bioassay replicates were obtained for each dosage of pooled or individual female fractions. Additionally, the female response to pooled female or male fractions was studied in a similar manner.

Data were evaluated by Student's t-test, linear regression and analysis of variance or covariance with significance being reported at the 0.05 probability level. Fraction numbers in this study refer to Sephadex G-25-40 chromatography unless otherwise noted.

Tentative molecular weight estimations were obtained following gel filtration on both Sephadex G-25-40 and Bio-Gel P-2 (100-200 mesh) using Blue Dextran 2000, synthetic oxytocin, oxidized glutathione, maltotriose, and para-nitrophenol as standards of known molecular weight. Glucose-free Tyrode's solution was used as an elutant. Standards were detected by their 280 μm absorbance on a Beckman 24:25 spectrophotometer or by visible color after a Lowry's or Benedict's procedure. Bio-Gel procedures were similar to those previously stated for Sephadex filtration.

RESULTS

Pheromone diffusion

The bioassay results of the experiment in which male responders were exposed to a zero vs 2-hour pheromone gradient are shown in figure 1. Simultaneous placement of the responding males and pheromone in the assay chamber caused a reduced level of response during the last two hours of the response period (after 2 hours of gradient formation) when compared to male responses to an established 2-hour gradient. However, both assays showed a significant dosage-response relationship during the third and fourth hours of gradient formation. Little male response was evident in the first two hours of gradient formation in the simultaneous placement bioassay. Maximal locomotor response to female sex

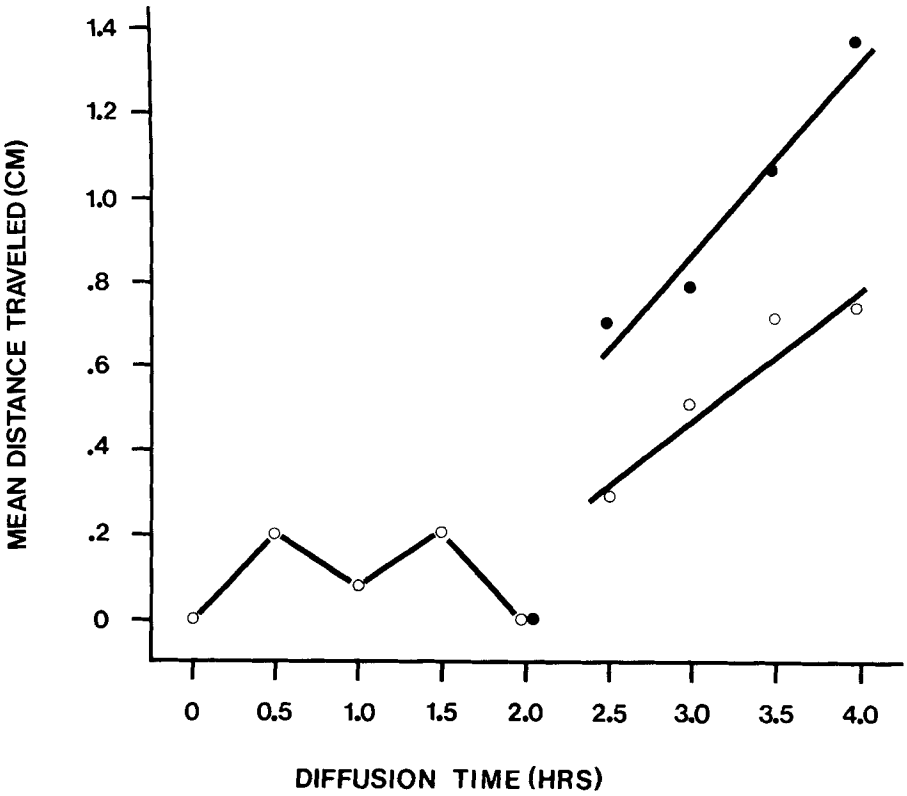

Fig. 1 Mean distance traveled by male *Nippostrongylus* toward the source of a 400 female-hour pheromone gradient that was introduced into bioassay chambers at the same time as the responders (○) or two hours earlier (●).

pheromone by male *Nippostrongylus* in this in vitro bioassay chamber apparently requires a critical period for gradient formation.

Thermal lability

The thermal lability and specific activity of pheromone derived from female *N. brasiliensis* are given in table 1. The crude pheromone derived from female incubate apparently is labile to moderate heat and freezing before purification. Microbial decomposition through direct degradation or through indirect alteration of other physiochemical parameters is weakly suggested at temperatures conducive to microbial growth, although no significant differences were found among the sterile vs non-sterile preparations at the three tested temperatures nor were significant differences found for the various temperature treatments other than inactivation after storage at −20°C for ten days. However, it appears intuitively obvious that low, though above freezing, temperatures should preserve pheromonal activity.

Male response to female fractions

Significant dosage-dependent bioassay responses were found for pooled column fractions at K_{av} 0.64 (F^4_{99} = 3.32) and K_{av} 1.0 (F^4_{99} = 5.44) (figs. 2, 3). Other pooled female fractions failed to yield a significant dosage-dependent attraction response by male *Nippostrongylus* and occasionally revealed highly erratic positive or negative responses.

Male response to individual active female fractions

The response of male *Nippostrongylus* to 60 female-equivalent dosages from individually assayed fractions within the active chromatographic regions is given in figures 4A and B. Fraction 13 from the K_{av} 0.64 pool was the only attractive sample in this group (fig. 4A). The +0.4 cm response to this fraction coin-

TABLE 1

Specific activity of non-sterile and sterile female sex pheromone incubate of N. brasiliensis *after storage as determined by male bioassay response*

Temperature (°C)	Storage (days)	Mean distance traveled (cm) by males toward pheromone source (±S.E.)	Relative specific activity (%)[1]
−20, non-sterile	1	+0.45 (±0.19)	36
1, non-sterile	1	+0.7 (±0.33)	56
4, non-sterile	1	+0.725 (±0.19)	58
22, non-sterile	1	+0.45 (±0.29)	36
37, non-sterile	1	+0.3 (±0.11)	24
4, sterile	1	+0.45 (±0.13)	34
22, sterile	1	+0.8 (±0.21)	64
37, sterile	1	+0.425 (±0.19)	34
−20, non-sterile	10	+0.1 (±0.26)	8

[1] Previously reported dosage-response data predict fresh 400 female hours should elicit a +1.15 cm male response (100% specific activity).

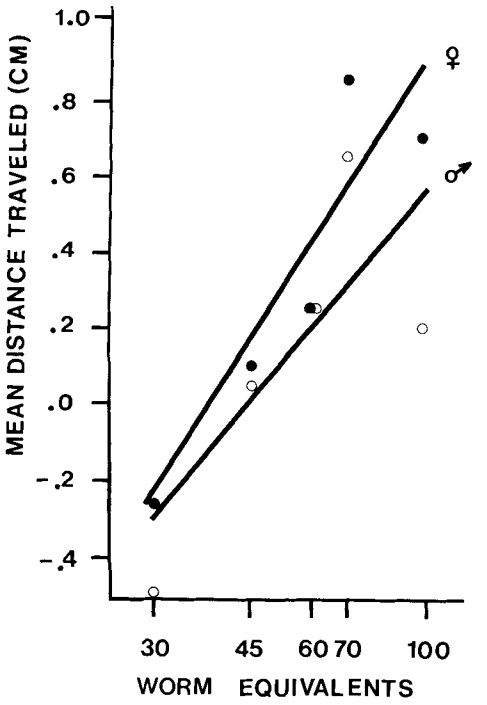

Fig. 2 Mean distance traveled by male *Nippostrongylus* to female (●) or male (○) worm equivalents of fractions at K_{av} 0.64.

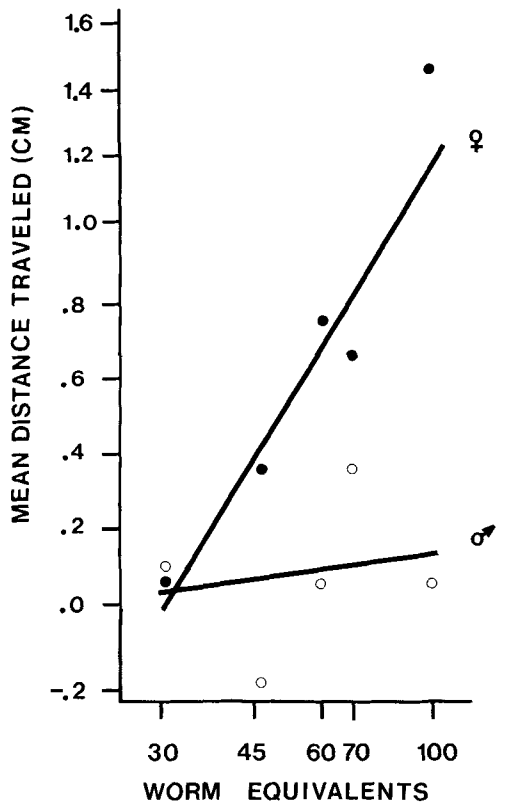

Fig. 3 Mean distance traveled by male *Nippostrongylus* to female (●) or male (○) worm equivalents of fractions at K_{av} 1.0.

cides with the +0.44 cm response predicted by the regression line (fig. 2) for the pooled K_{av} 0.64 fraction at 60 female equivalents.

Within the K_{av} 1.0 pooled sample, fractions 18 and 20 tended to be slightly attractive to male *Nippostrongylus* while fraction 19 was the most active. The summation of the activity in fractions 18, 19, and 20 (+0.13, +0.40, and +0.23 cm, respectively) represents a response of +0.76 cm which is identical with the +0.76 predicted by regression analysis (fig. 3) for 60 female-equivalents.

These data indicate that the K_{av} 0.64 fraction exhibits a narrow band of attractive activity for male *Nippostrongylus* while the pooled K_{av} 1.0 fractions contain a broader band of activity with the same degree of activity occurring in fraction 19 as was found in fraction 13. As expected from the gel properties, peaks became broader as the inclusion volume of the column was approached.

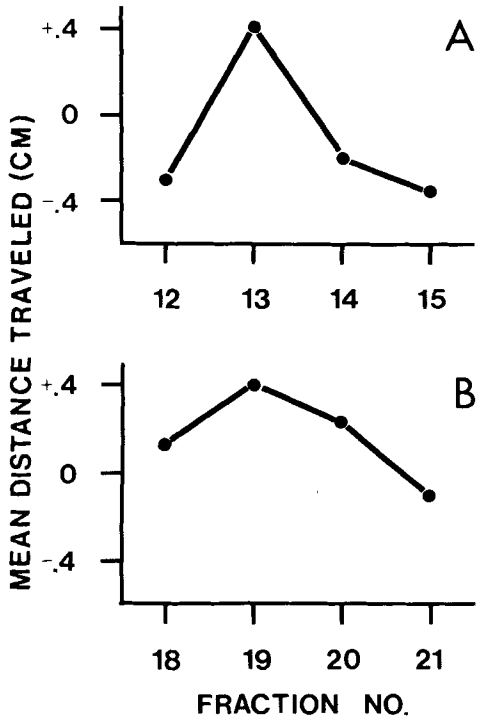

Fig. 4 Mean distance traveled by male *Nippostrongylus* to individual fractions from within the active female fractions 12-14 (K_{av} 0.64) (A) and 18-20 (K_{av} 1.0) (B).

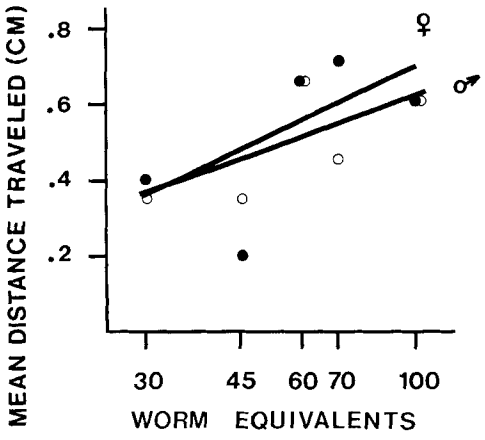

Fig. 5 Mean distance traveled by female *Nippostrongylus* to female (●) or male (○) worm equivalents of fractions at K_{av} 0.64.

Male response to male fractions

The response of male helminths to various male-equivalent dosages obtained from a similar chromatographic separation was also examined. Dosage-response lines were obtained for each pooled male fraction as for the female pheromone sources. Pooled K_{av} 0.64 male fractions elicited a significant dosage-dependent response (F^4_{99} = 3.17) from male *Nippostrongylus* (fig. 2). Covariance analysis indicated that no significant differences existed between the regression lines for the male response to male or female K_{av} 0.64 fractions. Based on these results, compounds are eluted in this region that are present in both sexes and are equally attractive to *Nippostrongylus* males.

Pooled male K_{av} 1.0 fractions were also assayed (fig. 3). Dosage-response effects were absent (F^4_{99} = 0.08) at the tested levels and no response was significantly different from zero. The regression of male response to K_{av} 1.0 fractions from males was significantly different in slope from that from females. These results indicate that an active compound(s) is found in fractions at K_{av} 1.0 of female helminths only.

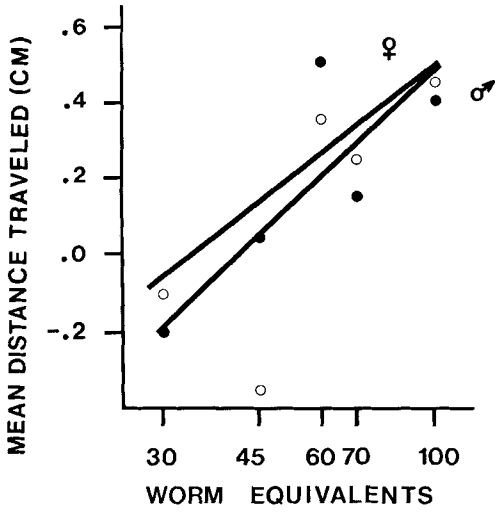

Fig. 6 Mean distance traveled by female *Nippostrongylus* to female (●) or male (○) worm equivalents of fractions at K_{av} 2.7.

Female response to male fractions

No male fraction was found to elicit a significant dosage-dependent female response, possibly because of the shallow slope of the calculated regression lines. However, female *Nippostrongylus* were significantly attracted to the 60, 70, and 100 male-equivalent dosages from K_{av} 0.64 fractions (mean SE = ± 0.42) (fig. 5). Additionally, females were significantly attracted to the 60 and 100 male-equivalent dosages of male K_{av} 2.7 fractions (mean SE = ± 0.35) (fig. 6). A dosage-dependent response may occur at higher dosage levels.

Female reponse to pooled female fractions

Female helminths exhibited a significantly dosage-dependent response to K_{av} 2.7 fractions (fig. 6). None of the remaining pooled female fractions caused a dosage-dependent response by female worms. However, female responses to 60, 70, and 100 female equivalents of K_{av} 0.64 fractions were significantly different from zero (mean SE = ± 0.40) (fig. 5).

There was no significant difference between the female response to male or female K_{av} 0.64 fractions according to analysis of covariance (fig. 5). The female response to male or female K_{av} 2.7 fractions also revealed no significant difference in attractiveness (fig. 6).

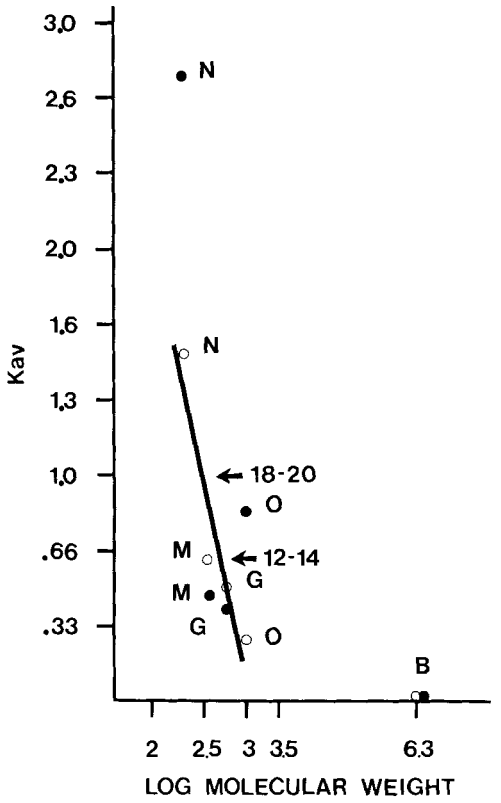

Fig. 7 Elution of molecular weight standards on Sephadex G-25-40 (●) or Bio-Gel P-2 (○) (B, Blue Dextran; G, gluthathione-oxidized; M, maltotriose; N, paranitrophenol; O, oxytocin). Female fractions 12-14 (K_{av} 0.64) and 18-20 (K_{av} 1.0) on the Bio-Gel column are indicated by arrows. (K_{av}, elution vol.–void vol./total vol.–void vol.).

Molecular weight estimations

Calibration of Sephadex G-25-40 and Bio-Gel P-2 columns with known molecular weight standards is shown in figure 7. K_{av} 0.64 fractions, compound(s) which is found in both sexes and which causes aggregation of both sexes, were found to elute slightly later than maltotriose (molecular weight = 504.5) on Sephadex, which suggests a molecular weight over 500. K_{av} 1.0 fractions, the probable female sex pheromone of *Nippostrongylus*, which causes locomotion responses by males only, exhibits a molecular weight under 400 on Sephadex. However, the delayed elution of the oxytocin standard indicated that collaboration on another matrix (Bio-Gel) was necessary.

K_{av} 0.64 fractions co-chromatographed with maltotriose on Bio-Gel, thus supporting an approximate weight over 500. K_{av} 1.0 fractions were again eluted somewhat later and based on the calibration line exhibit a molecular weight slightly under 400 as determined on Sephadex. Both Sephadex G-25 (a polydextran gel cross linked with epichlorohydrin) and Bio-Gel P-2 (a polyacrylamide gel) separate molecules based on their Stoke's radii. The similar retention volumes of the active fractions on these chemically different gel permeation columns indicate slight interaction of the active components with the permeation matrix. Molecular weight estimates assume a globular configuration and no gel-compound interaction. Thus, our suggested molecular weight are considered only tentative and are probably more useful for designing purification procedures than for absolute weight assignments.

DISCUSSION

The diffusion period required in our bioassay for sufficient gradient establishment to allow directional locomotory response

by the assay worms is approximately two hours. A reduction in the response caused by continuous exposure of males in the chambers during the period of pheromone gradient establishment may result from sensory adaptation to the pheromone. Unpublished observations indicate that a four-hour diffusion period also appears to reduce the male pheromone responsiveness, probably due to equalization of pheromone concentration throughout the assay apparatus.

Based on thermal studies, the female pheromone obtained from incubation of *Nippostrongylus* is short-lived in vitro and probably in vivo. Green ('66) also suggested the *Heterodera* female sex pheromone lost activity rapidly. This loss of activity may reduce the possibility of false gradients and thus prevent male confusion (Green, '66). Low, but above 0°C, temperatures apparently are required to preserve experimental levels of activity of the *Nippostrongylus* female pheromone.

Our chromatographic data apparently clarify portions of our and other earlier studies on the pheromone biology of *Nippostrongylus*. The sexes of *Nippostrongylus* exhibit attraction to three compounds (or groups of compounds) based on our interpretation.

K_{av} 0.64 fractions may represent an aggregation pheromone for male and female helminths that is produced by both sexes. Female-to-female attraction in *Nippostrongylus* was initially reported by Roberts and Thorson ('77). Bone et al. ('77) reported a low level attraction of female *Nippostrongylus* to males, while Bone and Shorey ('77) found female-to-female attraction which was not significant. We feel our earlier reports and that of Roberts and Thorson ('77) regarding attraction of males and females to females and attraction of females to males may result from K_{av} 0.64 fractions. The function of this compound(s) remains unknown.

K_{av} 1.0 fractions may represent the only true sex pheromone involved in premating attraction between the sexes of *Nippostrongylus*. We believe this fraction is largely responsible for the male and female attraction in our studies, although the role of K_{av} 0.64 fraction cannot be discounted.

Attraction in *Nippostrongylus* is shown also by females in their tendency to move toward the source of K_{av} 2.7 fractions. This chromatographic region has a molecular weight less than 140. Thus, the slight female response may indicate simple chemotaxis to a variety of cations, anions or other solutes. A comparison could be made between female attraction to K_{av} 2.7 fractions and Stringfellow's ('74) report that the hydroxyl ion served as a pheromone for *Pelodera strongyloides*.

The work of other investigators supports our localization of two active fractions with differing behavioral effects. The report of Balakanich and Samoiloff ('74) is especially encouraging. Balakanich and Samoiloff found two main chromatographic fractions from female *Panagrellus* that were attractive. A high molecular weight fraction attracted and retained males and females. Based on behavioral activity and elution characteristics, this *Panagrellus* fraction may coincide with *Nippostrongylus* fractions at K_{av} 0.64. Additionally, Balakanich and Samoiloff ('74) reported a male-attractive female fraction that eluted at 60-70% of the inclusion volume. This fraction appears remarkably similar in activity and elution characteristics to *Nippostrongylus* female K_{av} 1.0 fractions. These similarities might prove widespread as investigations of nematode pheromones increase and offer definite advantages in isolation and identification of the compounds. Although gel filtration yields only crude estimates of molecular weights, our studies indicate that the active components are small enough to be possibly identified and synthesized with existing methodology.

ACKNOWLEDGMENTS

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