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Instar Susceptibility of *Simulium vittatum* (Diptera: Simuliidae) to the Entomogenous Nematode *Neoaplectana carpocapsae*[1]

Randy Gaugler and Daniel Molloy[2]

Abstract: Laboratory bioassays showed that the susceptibility of *Simulium vittatum* to *Neoaplectana carpocapsae* increased with successive larval instars. First, second, and third instar larvae were resistant to infection, while seventh instars were highly susceptible. Significant differences in intra-instar susceptibility were also evident, as mortality ranged from 58% for the smallest seventh instar larvae to 97% for the largest. Dissections revealed that the basis for the resistance of early instars was physical exclusion of the comparatively large nematodes. The principle factor regulating the susceptibility of mid and late instars was injury to nematodes caused by larval mouthparts during ingestion. Differences in intra-instar susceptibility were similarly related to nematode injury. *Key words:* biocontrol, black flies.

The susceptibility of insects to control agents has generally been found to decline with increases in insect size (age). This principle has been demonstrated with chemical insecticides (2), as well as for biological agents such as entomopathogenic viruses (1), bacteria (8), and mermithid nematodes (12). Exceptions, however, are not uncommon; in tests with the entomogenous nematode, *Neoaplectana carpocapsae,* against pine weevil larvae, Pye and Burman (13) reported no differences in instar susceptibility. In preliminary studies examining the pathogenicity of this nematode to black fly larvae, we found that, on the basis of eye-selected size, early instar *Simulium verecundum* larvae were resistant and late instar larvae were highly susceptible to the nematode (9).

The present study provides detailed information on the comparative inter-instar and intra-instar susceptibilities of the seven larval instars of *S. vittatum* to *N. carpocapsae* and examines the factors governing larval susceptibility.

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[2]Biological Survey, New York State Museum, State Education Department, Albany, NY 12230. We thank S. Wraight and H. Jamnback for their critical review of this manuscript and T. Haskins for valuable technical assistance. This work was supported by NIH Grant 1R01AI1 5605-01.

MATERIALS AND METHODS

The DD-136 strain of *N. carpocapsae* was reared in larvae of the greater wax moth, *Galleria mellonella,* according to the technique of Dutky et al. (5). Infective stage juveniles were passed through nylon mesh filters before use to ensure inoculation with viable nematodes only. Black fly larvae were collected in the field from a mixed instar population of *S. vittatum* (IS-7 sibling species). Differences in the number of larvae tested at each instar reflect the proportions naturally present at the time of collection.

Tests were conducted in bioassay units which used pumps to recirculate stream water from reservoir tubs up to downward sloping effluent lips (6). A smooth laminar sheet of water flowed over each lip at a rate of about 8 liters/min. Larvae were introduced onto the lip portion of each unit and allowed 24 h to attach and acclimate before testing. Host-parasite interactions were observed through the thin film of flowing water with a stereomicroscope. Stainless steel screens (300-μm mesh size) prevented escape of mid and late instar larvae. Detaching early instars (<1%) were captured on organdy filters (200-μm mesh size) and returned to the lips. Temperature was maintained at 20 $\pm$ 2 C with aquarium heaters.

The instar susceptibility of *S. vittatum* to *N. carpocapsae* was evaluated at an initial

concentration of 5.0 juveniles/ml over a 15 min exposure period. This treatment was replicated 12 times with an average of 170 mixed instar larvae per replicate lip; controls were replicated six times. Treatment was ended by filtering nematodes from the test water with a cartridge filter (1.6 μm pore size) and pump. This method quickly removed all circulating nematodes, but ingested juveniles which did not successfully invade a host were subsequently egested back into the test water. Thus, nematodes were continually circulated during the test at extremely low concentrations (<.001/ml). Immediately after treatment 15 mg/liter of stream detritus was added to each bioassay unit to replace naturally occurring food particles removed by filtration. The experiment was ended 72 h after treatment, and all larvae were preserved for instar determination. The larvae were then classified by postgenal (part of the head capsule) length into seven groups corresponding to the seven larval instars of *S. vittatum,* as determined by the frequency distribution of Ross and Merritt (14). Although our data fit well within their distribution, postgenal length can vary with locality, so additional characters, including the degree of development of the pupal respiratory organ, were used to confirm this scheme. The larval instars and respective postgenal lengths were as follows: first, $\leq$100 μm; second, 101–140 μm; third, 141–200 μm; fourth, 201–280 μm; fifth, 281–380 μm; sixth, 381–480 μm; seventh, $\geq$481 μm. Seventh instars were further divided, according to postgenal length, into five groups to determine intra-instar differences in *S. vittatum* susceptibility. For purposes of convenience, first and second instars are referred to here as early instar larvae, third-fifth as mid instars, and sixth and seventh as late instars. The susceptibility of pupae was tested at a dosage of 100 nematodes/ml with continuous exposure for 3 d.

A second series of exposures was designed to assess the fate of ingested nematodes. Procedures were as described above, except that immediately after treatment larvae were transferred to a beaker of water maintained at 4 C to await dissection. All dissections were completed within 2 h of treatment. After postgenal length measurement, larval gut contents were examined under the stereomicroscope and nematode presence and viability recorded. Dead nematodes were easily recognized as they remained motionless even after prodding and had an opaque rather than a clear esophageal region. Gut contents of early and mid instar *S. vittatum* were further inspected under the compound microscope for the presence of nematode fragments. Remaining larval tissues were also examined to ascertain whether any nematodes had penetrated into the hemocoel. This test was repeated nine times at initial nematode concentrations ranging from 2.1–40.0/ml. Data from the nine treatments were pooled since no differences were apparent among treatments.

Data used in regression analysis were transformed to arcsin and weighted by sample size (n_1).

RESULTS AND DISCUSSION

A direct relationship (r = 0.97) was noted between larval instar and larval susceptibility to *N. carpocapsae* (Table 1). Mortality increased progressively, from 0% for first-third instars to 75% for seventh instar larvae. There were also significant intra-instar differences in susceptibility: mortality among seventh instars ranged from 58% for the smallest larvae to 97% for the largest (Table 2). The high susceptibility of last instars was further demonstrated by our observation that only two

Table 1. Inter-instar susceptibility of *Simulium vittatum* larvae to infective stage juveniles of *Neoaplectana carpocapsae.*

Instar	No. larvae dead/ treated	Corrected percentage mortality* ± SE	95% confidence limits
I	0/6	0	
II	0/20	0	
III	1/105	0	
IV	13/257	1.3 ± 0.95	0- 3.2
V	116/591	16.4 ± 3.05	9.5-23.3
VI	177/424	38.6 ± 4.27	28.9-48.3
VII	227/290	75.3 ± 3.23	69.0-81.6

*Corrected by Abbott's formula with control mortality: I, 0% (0/2); II, 0% (0/11); III, 6.5% (4/62); IV, 3.8% (5/132); V, 3.9% (14/357); VI, 5.2% (18/347); VII, 12.0% (49/408).

Table 2. Intra-instar susceptibility of last instar larvae of *Simulium vittatum* to infective stage juveniles of *Neoaplectana carpocapsae.*

Larval postgenal length (μm)	No. larvae dead/ treated	Corrected percentage mortality* ± SE	95% confidence limits
481-499	50/77	57.9 ± 8.10	39.6- 76.2
500-524	41/53	75.1 ± 5.92	63.5- 86.7
525-549	82/104	74.5 ± 5.62	61.8- 87.2
550-574	23/24	95.5 ± 4.25	87.2-100.0
≥575	31/32	96.9 ± 3.38	90.3-100.0

*Corrected by Abbott's formula with control mortality: 481-499 μm, 16.7% (14/84); 500-524 μm, 9.0% (10/111); 525-549 μm, 17.2% (20/116); 550-574 μm, 8.3% (5/60); ≥575 μm, 0% (0/37).

treated larvae pupated (0.7% of the treated last instars), compared to 31 control larvae (7.1% of the control last instars). Pupae were not susceptible to infection even under conditions of prolonged exposure at high nematode concentrations. Since infection of black flies by *N. carpocapsae* in flowing water occurs only through ingestion, the nonsusceptibility of the pupal stage was not unexpected. Because a greater proportion of surviving than dead larvae could potentially have molted during the 3-d test period, the mortality reported here probably is slightly underestimated. These results confirm our previous preliminary observations that late instar black fly larvae are more susceptible than early instars (9) and demonstrate the close relationship between insect size and both inter-instar and intra-instar susceptibility.

Black flies, like most insects, tend to become more tolerant to control agents with successive instars. Conventional insecticides, for example, are generally most effective against early instars (7,15), as are entomopathogenic bacteria (10) and mermithid nematodes (11). Thus, *N. carpocapsae* is conspicuous for its greater efficacy against late instar larvae.

Wallace et al. (15) have suggested that the different susceptibilities of black fly larval stages to toxicants may be due to the different feeding rates and physiological tolerances among instars. In our initial investigation (9) we suggested that the resistance of early instar *S. verecundum* was a result of the disproportionate size between these larvae and the nematodes. Microscopic observations in the present study have shown that the tolerance of early and mid instars is indeed related to their inefficiency in capturing and ingesting juveniles. Most captured juveniles were lost in transfer from the cephalic fans to the cibarium. Dissections of treated larvae confirmed that physical exclusion was the basis for the resistance of first and second instars (Table 3). Evidently, these instars are too small to engulf the 25- × 550-μm infective juveniles, as not even nematode fragments were recovered from their guts. In contrast, sixth and seventh instars readily ingested several hundred juvenile nematodes each, the number ingested appearing to be limited only by gut capacity. Mid instars usually contained 2–50 juveniles, depending on larval size. Most juveniles were found contained within the peritrophic membrane. Those found in the hemocoel were usually in the anterior portion of the body, suggesting that penetration occurs through the foregut as with mosquito larvae (16).

Table 3. The effect of *Simulium vittatum* larval instar on the ingestion and subsequent viability of infective stage juveniles of *Neoaplectana carpocapsae.*

Instar	No. of larvae examined	Percentage (No.) of larvae: Without juveniles	With juveniles: Dead only	With juveniles: ≥ one viable	Average percent juvenile viability ± SE
I	20	100.0 (20)		0 (0)	
II	20	100.0 (20)		0 (0)	
III	35	65.7 (23)	28.6 (10)	5.7 (2)	11.5 ± 8.63
IV	46	34.8 (16)	52.2 (24)	13.0 (6)	6.7 ± 2.85
V	77	6.5 (5)	51.9 (40)	41.6 (32)	9.2 ± 1.65
VI	76	0 (0)	6.6 (5)	93.4 (71)	33.7 ± 3.29
VII	86	0 (0)	0 (0)	100.0 (86)	79.1 ± 2.67

The susceptibility of mid and late instars was strongly correlated (r = 0.99) with injury to juveniles caused by larval mandibles during ingestion (Table 3). For example, more than half (52%) of all fourth instar larvae examined contained only injured (dead) nematodes. Since an additional 35% excluded juvenile entry entirely, only 13% of fourth instars could conceivably have become infected, because only that percentage ingested one or more viable (uninjured) juveniles. By comparison, 6.6% of sixth instar larvae contained injured juveniles only, and exclusion did not occur, so that it was possible for 93.4% to become infected. This resulted, however, in 38.6% mortality (Table 1), not 93.4%, since the average viability of juveniles contained in these larvae was only 34%. Similarly, 41.6% of treated fifth instars ingested one or more viable nematodes, but mortality was a mere 16.4% (Table 1) because less than 10% of the juveniles were viable. Ingestion of a viable nematode obviously does not insure that infection and mortality will result. Larval mortality may be presumed to be related to the number of viable nematodes ingested, with an undetermined minimum number required for mortality to occur.

The extent of juvenile injury was similarly related to larval size; i.e., the smaller the black fly the more damage to the nematode. Third instars often contained only nematode fragments or nearly empty cuticles, while seventh instars inflicted limited damage, leaving the juvenile intact but rupturing the intestine so that its contents filled the pseudocoelom. More than 90% of the viable nematodes recovered from seventh instars were found to be exsheathed, while most (>95%) dead juveniles were still ensheathed.

Intra-instar differences in susceptibility were also largely attributable to injury caused to juveniles by larval mouthparts. Dissections of seventh stage larvae, all of which ingested viable nematodes, showed that viability increased from 45 to 94% as larval size increased (r = 0.96) (Table 4). Juvenile viability in these larvae was similarly closely correlated with susceptibility (r = 0.99).

In experiments wth the mosquito, *Culex pipiens,* Dadd (4) also observed that larval size excluded *N. carpocapsae* ingestion by early instars and that some injury to juveniles occurred during ingestion. Dadd further reported that viable juveniles remaining within the gut were rapidly degraded, undergoing "total dissolution" within 24 h. He postulated that cuticle lesions caused during ingestion might have permitted the attack of digestive enzymes. We suspect that because of the high internal pressure of nematodes (3), such lesions would likely have caused immediate nematode rupture. Despite extensive examination we did not observe viable juveniles to be digested by black fly larvae. No alternative hypothesis is offered for Dadd's observation.

Table 4. Viability of infective stage juveniles of *Neoaplectana carpocapsae* following ingestion by last instar larvae of *Simulium vittatum.*

Larval postgenal length (μm)	No. of larvae examined	Average percent juvenile viability ± SE
481-499	7	45.3 ± 4.64
500-524	11	61.2 ± 8.46
524-549	19	65.1 ± 5.22
550-574	24	89.3 ± 3.94
≥575	25	93.8 ± 2.58

It is evident from our results that differences in inter-instar and intra-instar susceptibility are ultimately due to differences in larval cibarium size and not feeding rates, physiological factors, larval weight, or other intrinsic factors. Cibarium size would regulate both the exclusion and injury of infective juveniles. The observed differences in intra-instar susceptibility alone are convincing evidence that susceptibility is a function of larval size.

The effective use of *N. carpocapsae* against black flies appears to be mostly limited to treatment of late instar populations, as mid instars are only moderately susceptible and the resistance of early instars could not be overcome by high dosages. This limitation would require close monitoring of larval populations to determine the optimal time of applications; i.e., shortly before pupation when the larvae are most susceptible. Treatment of asynchronous populations would require the integration of infective juveniles with alternative control agents,

most of which show their greatest efficacy against early instars.

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