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## Larval rearing conditions affect kin-mediated cannibalism in a treehole mosquito

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Cannibalistic behavior among *Ochlerotatus triseriatus* larvae was studied to determine whether cannibals are able to alter their attack behavior based on their relatedness to newly hatched conspecific prey larvae. Fourth instar larvae, reared in one of four different initial densities, were placed with newly emerged first instars and, after 48 h, the number of first instar larvae remaining was recorded. Our data suggest a Type III functional response of non-kin fourth instar larvae to first instar prey density. Several significant effects emerged from our analysis model, namely that the number of first instar prey available, the relationship of the first instar larvae with the cannibals, and the density at which the fourth instar cannibals were reared all affected the number of first instar larvae consumed.

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Cannibalism is a common form of predation in nature (Fox 1975, Polis 1981, Elgar and Crespi 1992). Several evolutionary consequences of cannibalism have been proposed. Some suggest that cannibalism is favored by natural selection because of direct metabolic gain from eating conspecifics and indirect gain from reducing the number of competitors (Polis 1981, Wagner et al. 1999). Other arguments contend that cannibalism is not favored by natural selection because sibling cannibalism may reduce the inclusive fitness of the cannibal (Hamilton 1964), cannibals may suffer increased mortality from being injured by conspecific prey (Polis 1981), or cannibals may be infected by pathogens contracted from eating diseased conspecifics (Pfennig et al. 1991). Since cannibalism does occur frequently in the wild, one would expect that it is favored by natural selection, and that compensatory mechanisms exist to reduce the costs relative to the benefits. These compensatory mechanisms may include kin-avoidance, attenuated prey selection and diseased-conspecific evasion. Since studies of kin-avoidance and kin-recognition have produced contradictory results, especially among non-

social insects, we investigated the prevalence of kin-avoidance among kin and non-kin groups of the mosquito *Ochlerotatus triseriatus*.

Many authors have reported kin-biased behavior with respect to cannibalism among non-social insects. For example, Wade (1980) reported that flour beetle larvae cannibalized non-kin eggs in preference to sibling eggs. Nummelin (1989) found that adult waterstriders consumed non-kin nymphs more often than kin nymphs. For the ladybird beetle *Adalia bipunctata*, females and second instars avoided eating their own or sibling eggs (Agarwala and Dixon 1993). Joseph et al. (1999) reported that larvae of the ladybird beetle *Harmonia axyridis*, consumed kin significantly less often than they consumed non-kin. Fellowes (1998) lists several examples of other organisms that appear to exhibit kin-biased behavior.

Other investigators have tested for kin-biased behavior with negative results. For instance, field experiments by Gastreich et al. (1990) showed that the wasp *Parachartergus colobopterus*, admitted conspecifics into its nest without discriminating between kin and non-

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kin. Aphid soldiers were shown to discriminate between soldiers and non-soldiers, rather than between kin and non-kin (Aoki et al. 1991). Among some mites, the level of aggression directed toward kin was not significantly different from the level of aggression directed toward non-kin (Radawan 1993). Another wasp, *Bracon hebetor*, avoided mating with brood-mates, but readily mated with siblings from a different host (Ode et al. 1995). Finally, Sherratt et al. (1999) found no evidence for kin discrimination in the cannibalistic treehole mosquitoes *Trichoprosopon digitatum* and *Toxorhynchites moctezuma*.

Several aspects of life history and behavior of the treehole mosquito, *Ochlerotatus triseriatus*, are useful for testing hypotheses regarding the organization of kin-biased behavior. These include larval cannibalism, erratic egg hatching, and larva-induced egg hatch inhibition. Larval cannibalism occurs in some mosquito species (Reisen and Emory 1976, Seifert and Barrera 1981, Koenekoop and Livdahl 1986, Annis et al. 1990, Sherratt and Church 1994, Church and Sherratt 1996, Lounibos et al. 1996), and furnishes a convenient system to test for kin-biased behavior. In some species, the eggs from a single batch laid by a single female hatch in installments over long periods (Gillett 1955a, b). One consequence of erratic hatching is that siblings from different developmental stages interact; thus, fourth instar larvae frequently share a habitat with their first instar siblings. Within mixed cohort groups of these mosquitoes, developmentally advanced larvae can prey upon their newly hatched conspecifics. In this situation, cannibals could be consuming their kin. Evidence from *Anopheles messeae* suggests that certain genotypes may result in more aggressive, cannibalistic larvae that display an overall more invasive phenotype than non-cannibalistic conspecifics (Gordeev and Troshkov 1990, Gordeev and Perevozkin 1997).

In this study, we assessed whether kin discrimination occurred among the larvae of the treehole mosquito, *O. triseriatus*. We hypothesized that the rate at which fourth instar larvae consume kin was less than the rate at which they consume non-kin. To test this hypothesis, we exposed fourth instar larvae either to first instar siblings or to non-siblings, and recorded the number of first instars consumed in the ensuing 48 h.

## Methods

We collected *O. triseriatus* larvae from treeholes and tires at four different locations around Worcester, Massachusetts, USA during the summer of 1998. All larvae were returned to the laboratory, reared in water-filled aluminum cake pans, fed ad libitum with rat food (Formulab® Chow 5008), and raised to the pupal stage. Pupae were removed and placed in Gerberg® mosquito

cages for emergence to adulthood. All adult mosquitoes were provided sugar water ad libitum, and the females were fed with blood from the principal investigator. Following blood feeding, we mated each adult female with a single male, then isolated the female in a covered 500-ml plastic cup, lined with cardboard, and filled with 100 ml of de-ionized water, to facilitate oviposition. To feed the females after mating, we placed a small vial stuffed with cotton and filled with sugar water in each oviposition cup. Within a week or two, females oviposited on the cardboard lining of the oviposition cup. We then collected and counted the eggs, placed them on damp paper towels, and stored them until we were ready to perform the experiments. In this experiment all eggs, larvae, pupae and adults were maintained in an incubator under LD 18:6 illumination at 20°C and 80% relative humidity.

Ten days before the first experiment, we divided in half each of 108 egg batches collected from isolated females. For each egg batch, one half was immediately exposed to a hatch stimulus to provide fourth instar larvae for the experiment. The other half was stored on damp paper towels to provide first instar larvae for later use. Fourth instar larvae were produced by inundating the eggs overnight in a solution of nutrient broth (Difco Laboratories®) and de-ionized water (1 g/L). Following the hatch of the first group of egg batches, we placed the resulting larvae into one of four treatment groups of various rearing densities. The treatment groups were 50 larvae/100 ml, 20 larvae/100 ml, 10 larvae/100 ml, and 5 larvae/100 ml. Thus, as they were being reared to the fourth instar, these larvae experienced a wide range of densities. Each treatment group was provided with 100 mg of rat food.

At ten days, after all larvae had developed into their fourth instar, we hatched first instar larvae from the remaining eggs of each egg batch. The first instar larvae were hatched using Novak and Shroyer's (1978) hatching tube technique. Each hour, we removed first instar larvae from the hatching tubes and placed them (number varied according to how many hatched) into 35-ml vials filled with de-ionized water. The experiment was initiated immediately by placing five, either sibling or unrelated, fourth instar larvae in each vial with the first instar larvae. Due to our inability to control the number of first instar larvae hatching, the prey density in each treatment varied, an aspect of this experiment that had interesting consequences. Forty-eight hours later, we counted the number of first instar larvae remaining in each treatment.

All missing first instar larvae were assumed cannibalized. Prior studies showed that no first instar larvae disappeared in the absence of older larvae and that the time period is too short to permit larval decomposition (Koenekoop and Livdahl 1986). Since the numbers of first instar larvae in the treatments varied, we calculated a per capita rate of cannibalism as the number of first

Table 1. Summary of a multiple regression analysis of attack rate per predator, incorporating linear terms for each main effect (prey density, predator rearing density, and kinship), and terms for each pair-wise and three-way interaction among those factors. The right column shows 95% confidence intervals for each regression coefficient using resampling methods. The confidence intervals were obtained from frequency distributions of regression coefficients built from 10 000 samples of 108 data drawn with replacement from the original data set, and those that do not include zero are shown in italics.

Source	Estimate	d.f.	SS	F	P(> F)	Resampled C.I. (95%)
Prey density (Prey)	0.0126	1,100	0.031	9.45	0.003	<i>0.0046–0.0221</i>
Predator rearing density (Npred)	0.0029	1,100	0.024	7.42	0.007	<i>0.0003–0.0058</i>
Kinship (Kin)	0.0843	1,100	0.011	3.34	0.071	–0.0122–0.1688
Prey × Npred	–0.0006	1,100	0.033	10.13	0.002	<i>–0.0012–0.0002</i>
Prey × Kin	–0.0163	1,100	0.020	6.25	0.014	<i>–0.0294–0.0047</i>
NPred × Kin	–0.0035	1,100	0.013	4.10	0.045	<i>–0.0082–0.0007</i>
Prey × Npred × Kin	0.0007	1,100	0.028	8.63	0.004	<i>0.0002–0.0015</i>

instar larvae cannibalized by each fourth instar larvae, hereafter 'attack rate'. A regression model was used to test for the effects of the experimental factors and interactions among them on the attack rate. These included the main effects prey density, predator rearing density, and kinship. Kinship was assigned a value of 0 for non-kin prey and 1 for sibling prey. The potential for violations of assumptions led us to analyze this regression model using resampling methods which do not assume normal distributions or equal variances among treatment groups. We used Resampling Stats® software (Simon 1990) to perform the regressions with the full model 10 000 times on the independent variables and their cross-products, drawn randomly in samples of 108 from the full data set of 108 observations. Frequency distributions of the 10 000 bootstrapped regression coefficients were used to obtain 95% confidence intervals for the coefficients.

## Results

Our results indicated that fourth instar larvae are able to discriminate and avoid consuming larval kin when placed together with their younger conspecifics, but only under certain conditions. This interaction was significantly attenuated by several other factors including the density in which the fourth instar larvae were reared, the number of first instar prey available, and the interactions of these terms with kinship (Table 1). The most important findings of our model were that the rate at which fourth instar larvae consume conspecifics depends significantly on the number of first instar prey available, and that there is a significant kin effect on cannibalism. Furthermore, fourth instar larvae exhibit a Type III functional response to non-kin first instar larvae, which is absent in the presence of kin (Fig. 1). There is also a significant interaction among the prey available, the genetic relatedness of the cannibals and prey, and the density at which the fourth instar larvae were reared. This is best seen by the difference in slopes between kin and non-kin prey, which was most marked when predators were reared at low densities (Fig. 1a,

b). The slope difference was not present for predators reared at 20 per 100 ml (Fig. 1c), and it was reversed for predators reared at the highest density, 50 per 100 ml (Fig. 1d).

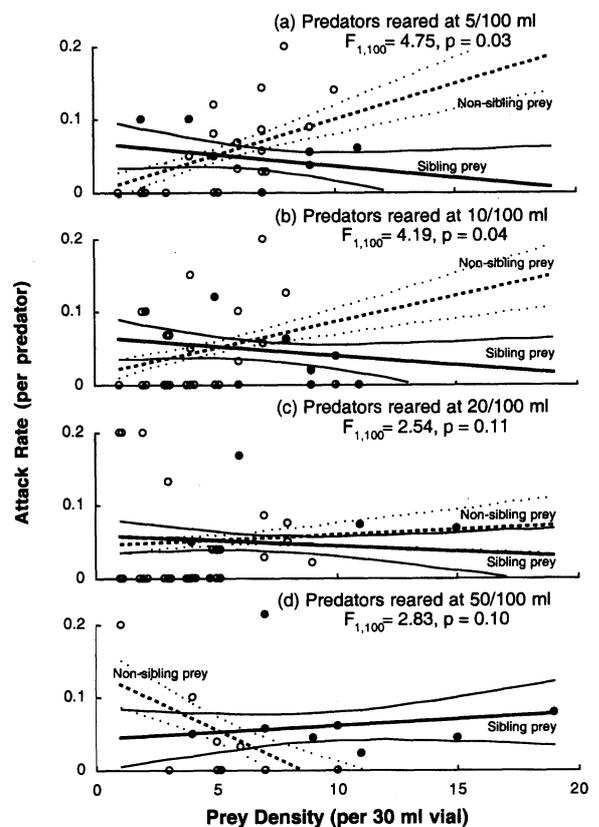


Fig. 1. Responses of attack rates (fraction attacked per predator) to the density of prey. Predators were reared at four different initial densities (a–d), and were exposed to either kin (full siblings, solid lines, closed circles) or non-kin (dashed lines, open circles). The predicted values of the regression model,  $\pm 1$  standard error of the predicted value, are shown with the observed data. For each predator rearing density, the difference between slopes for kin and non-kin prey was tested using the Prey × Kin interaction mean square for each subset of data and the mean square for the full data set. These results are shown for each rearing density.

## Discussion

A surprising result of our study was the positive response to prey density observed for non-kin, but not for kin, fourth instar larvae. For fourth instar larvae exposed to kin, the number of kin consumed remains constant even as the prey density increases. On the other hand, non-kin fourth instar larvae respond positively to increasing prey density by consuming more conspecifics, a type of functional response first described by Holling (1959). A closer inspection of the data suggests that this response resembles Holling's Type III functional response, with the diagnostic feature of reduced rates of cannibalism at low prey densities. One explanation for this type of response is that cannibals exhibit reduced hunting efficiency at low prey densities. Another non-exclusive explanation for the Type III response is that cannibals lack an adequate search image for prey at low densities and encounter rates; they may fail to learn to recognize and actively seek out conspecifics. Reinforcement of learned searching behavior may be more likely at high prey densities. We presume that the second characteristic, a reduced cannibalism rate at high densities as predators become satiated, would also occur, had we provided sufficient prey to enable satiation; however, there is an upper limit to the density of full-sibling larvae that can be present in any habitat, determined by maternal clutch size.

Cannibals reared at high densities and exposed to high numbers of prey consumed fewer non-kin than when reared at low densities with fewer prey (Fig. 1). At low rearing densities cannibals presumably have fewer interactions with conspecifics during development. The attenuated levels of cannibalism that were apparent as cannibal rearing density increased may be due to an informal rule of thumb: don't eat conspecifics that are familiar to you. Since larvae can discriminate kin from non-kin, this "familiarity" may be inherent among kin. Therefore, rearing density may have no effect on kin cannibalism because larvae can innately avoid attacking kin. Alternatively, among non-kin, the familiarity rule is density dependent because cannibals should perceive organisms that convey similar sets of sensory cues as organisms that should not be consumed. In a mosquito breeding system, where the majority of organisms in the developmental milieu may be siblings, these two operations of the rule of thumb would be reinforcing in natural situations. Both factors provide a strong selective force for survival at high densities and in a system with staggered development of offspring. There may be a high degree of relatedness among the individuals in a treehole because most females avoid ovipositing where larvae are present and, when they do oviposit, they tend to leave most their eggs in one location (Edgerly et al. 1998). Consequently, there are few oviposition events for a given

treehole, and the behavioral rule of thumb prevents cannibalism among related individuals.

This study illustrates that experimental conditions must be carefully considered, especially in the study of cannibalism. As our results showed, prey density and cannibal rearing density can, in interaction or separately, significantly affect results. Previous studies of kinship and cannibalism that failed to find significant results may have been flawed because either there was insufficient prey available or because cannibals were developmentally delayed and never grew large enough to consume conspecifics. Since cannibalism may be highly size dependent, perhaps the rate of cannibalism (and any associated kin effects) would have been more prominent if the cannibals were fed *ad libitum*, raised under low density conditions, and allowed to grow to full size. Investigators must also ensure that sufficient prey are available to cannibals in order to elicit kin effects. Without careful consideration of the conditions, kin effects may be difficult to discern.

The conditions presented to the larvae in this experiment were deliberately constructed to examine cannibalistic behavior. For this reason, we did not provide an alternative food supply during the trials, because we sought to maximize our ability to make comparisons between kin and non-kin prey vulnerability. There are some situations in which these conditions could apply to habitats of natural populations, e.g., water within a recently discarded automobile tire, or in a wide variety of other domestic containers. Food has also been found to be in limited supply in natural treehole fluid (Livdahl 1982). Densities of larvae used in the experiment fit within the range of those found in natural treeholes. The fourth instar density (167/litre) was higher than the geometric mean density observed in natural treeholes in the northeastern US, but less than about 30% of treeholes sampled in field surveys during June and July (Livdahl and Edgerly 1987); first instar densities exceed those used in this experiment quite routinely after spring rains (Livdahl unpubl.).

Future studies should investigate the effects of varying prey density and varying larvae rearing densities on the occurrence of cannibalism among natural populations. One aspect of this study that should be improved upon is that cannibals were not given a choice between kin and non-kin conspecifics due to the difficulty of distinguishing the two in an experimental system. Perhaps future studies could use molecular techniques to allow discriminating between larvae in a choice experiment. We feel that our general rule of thumb would still apply in such a system. That is, growing up at high density may make cannibalism less common. However, if "forced" to consume kin, the ability to distinguish kin from non-kin could be selectively favored. We predict that the attack rate of non-kin will be high at low rearing density and would decline as rearing density increases. The relative ratio of kin to non-kin consumption, however, should always be less than one.

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