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Title: Prey preferences of notonectids towards larval mosquitoes across prey ontogeny and search area

A short running title: Search area affects ontogenic prey preferences of notonectids

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Abstract

BACKGROUND: Predatory biological control agents can be effective natural means of managing pests, vectors and invasive species. However, the strength of predator-prey interactions can be regulated through context-dependencies that often remain unquantified. In particular, refuge effects can influence the efficacy of biological agents towards target species, and such effects are often driven by prey size and search area differences. In the present study, we thus quantify prey preferences of two predaceous notonectids, *Anisops breddini* and *Anisops sardeus*, towards four different aquatic larval instar stages of the medically-important mosquito *Culex quinquefasciatus* across variations in surface area and water depth.

RESULTS: Consumption rates differed significantly among the four larval sizes but not between the notonectids. Search area variations also elicited differences in consumption rates. Both predators tended to prefer second instar mosquito prey among surface area and water depth variations, whilst generally avoiding the largest (fourth instar) and smallest (first instar) prey instar stages. For both predators, differential selectivity traits were emergent across search area variations and water depth, with refuge effects for small prey generally greatest under intermediate-high depths with high surface areas. We thus demonstrate that predatory impacts of notonectids towards mosquito larvae differ significantly according to prey size, and likely peak at intermediate size classes.

CONCLUSION: Different mosquito size classes often coexist and compete, this selectivity has important implications for adult mosquito proliferations. Further, in ephemeral aquatic habitats where search areas and water depths are highly variable spatiotemporally, the efficacy of notonectids in controlling mosquito prey may differ substantially.

Keywords: prey selectivity, *Anisops*, container size, mosquito larvae, predator-prey

1 INTRODUCTION

Predation plays a crucial role in ecosystem structuring and functioning through trait- and density-mediated effects on prey populations.^{1, 2, 3, 4} Accordingly, predatory biological control agents can drive substantial population reductions of target organisms (e.g.⁵). Lentic aquatic systems are characteristic breeding sites of medically-important mosquitoes, and predaceous insects such as hemipterans, coleopteran larvae and odonate larvae are often the top predators in both natural and artificial ephemeral aquatic habitats.⁶ More than one billion people are infected every year and more than 700,000 people die from mosquito-borne diseases, including malaria, dengue, Zika, Japanese encephalitis and yellow fever, spread by *Anopheles*, *Aedes* and *Culex*.⁷ This necessitates the urgent development of effective control strategies. *Culex quinquefasciatus* Say, 1823 (Diptera: Culicidae), a member of the *Culex pipiens* complex, is an efficient vector of lymphatic filariasis, brancroftian filariasis, Rift Valley fever virus, St Louis encephalitis, Sandbis virus, and West Nile virus (see^{8, 9, 10}). Mosquito species within the *C. pipiens* complex are abundant and exhibit a worldwide distribution, and colonise a broad range of aquatic environments, including ponds, wetlands, paddy fields and artificial containers.^{11, 12, 13} Although many terrestrial and aquatic predators of mosquitoes have been identified,^{5, 14, 15} and biological control has been effective in field applications (e.g.¹⁶), context-dependencies, such as prey preferences and search area variabilities, may alter predatory interaction strengths towards target pests.

Most invertebrate predators (e.g. Hemiptera, Hymenoptera and Araneae) are generalists in terms of their feeding strategy, which may in turn reduce their biocontrol potential towards target prey.^{17, 18, 19} Nevertheless, generalist predators can still assist or augment efforts to

manage pest populations (see ²⁰). In particular, within ecosystems containing multiple prey types, generalist consumers often exhibit ratio-dependent switching propensities among prey according to their environmental availability. ^{17, 21} Further, in predator-prey dynamics, refugia is common in prey survival strategies. ^{22, 23, 24} Ontogenetic prey size-refuge may reduce predatory efficacies towards target prey which are of a size that is difficult to handle. ^{25, 26} However, search area variations in ecosystems may further mediate refuge effects, and in aquatic habitats these effects may be driven primarily by differences in surface area and water depth. ^{27, 28, 29} Reciprically, variations in total water volumes may be interpreted through differences in relative predator and prey densities; however, explicit consideration of depth and surface area factors may enable better quantification of emergent effects between these variables. More broadly, an optimum size of prey is often disproportionately consumed by a predator, ³⁰ and this is strictly related to body mass ratios between predator and prey. While the consumptive associations of such body mass ratios have been extensively studied, ^{31, 32, 33} the relationship between prey size preferences and differences in search area within 3D aquatic environments have yet to be examined comprehensively in the context of pest management. In a mosquito biocontrol context, it is preferable that predators can handle and preferentially select late instars of mosquito larvae to avoid compensatory reductions in competition. ³⁴

Notonectids are voracious predatory insects which can play a key role in the structuring and functioning of aquatic ecosystems (e.g. ^{35, 36, 37}). In the tropics, notonectids often dominate both natural and artificial ephemeral aquatic ecosystems, where, in the absence of predators, mosquitoes can successfully develop and proliferate. ^{38, 39} Whilst their diet includes all instar stages of mosquito larvae, predatory efficiencies can differ according to the mosquito larval ontogenic stage. ⁴⁰ In particular, differential motility rates and prey sizes can affect predator-prey

encounter rates and, in turn, modulate interaction strengths.^{41,42} Although the predatory potential of notonectids towards mosquito larvae has been well-described (e.g.^{43,44,45,46}), it is unclear how variabilities in prey preferences and search area interact to alter the predatory impact of such consumers. This is particularly important for larval mosquito prey, which forage throughout the water column and exhibit rapid growth through ontogenic progression (see^{47,48}). Given examinations of biotic interactions for pest management in aquatic ecosystems often fail to address context-dependencies, such as water depth and surface area variations, alongside prey-size refuge effects, the present study investigates the prey preferences of two common notonectid predators, *Anisops breddini* Kirkaldy, 1901 (Heteroptera: Notonectidae) and *Anisops sardeus* Herrich-Schaeffer, 1849 (Heteroptera: Notonectidae), towards four larval instar stages of *Culex quinquefasciatus* to decipher the systematic effects of different container depths and surface areas on prey selectivity traits.

2 MATERIALS AND METHODS

2.1 Collection and maintenance of predators and prey species

Size-matched focal predators, adult *A. breddini* (7.05 ± 0.02 mm) and *A. sardeus* (7.25 ± 0.05 mm) were collected from fishery ponds in Irongmara, Cachar, Assam, India ($24^{\circ}41'15.78''\text{N}$, $92^{\circ}45'12.21''\text{E}$) during the summer-monsoon season of 2017 by trawling a net (mesh size 60 μm) through the water column. Notonectids were transported in source water to a laboratory in the Department of Ecology and Environmental Science, Assam University, Silchar. This laboratory was maintained at 30°C ($\pm 2^{\circ}\text{C}$) and under a 12:12 light:dark photoperiod. Both

predator species were acclimatized separately in a glass aquaria holding 25 L de-chlorinated tap water with continuous aeration for at least one week prior to the experiment. Water was changed every 10 days and notonectids were fed *ad libitum* with mosquito and chironomid larvae.

The prey, *C. quinquefasciatus* larval instars (first – fourth), were collected on-site within the Department of Ecology and Environmental Science, Assam University campus by sampling egg rafts from container-style habitats filled with a jaggery (a traditional non-centrifugal organic cane sugar lump made from date or palm tree; Arya Farm Products Pvt. Ltd) and water mixture to attract oviposition. The mosquito egg rafts were then transferred to the same laboratory as the notonectids. Upon hatching, mosquito larvae were reared and fed *ad libitum* with crushed jaggery to obtain the desired size classes. Four different instar stages of *C. quinquefasciatus* (mean length $\pm$ SE: first instar, 1.11 ± 0.04 mm; second instar, 2.21 ± 0.02 mm ; third instar, 3.98 ± 0.05 mm; fourth instar, 4.90 ± 0.03 mm) were used in the experiment.

2.2 Prey preference experiment

We employed ‘ $3 \times 3 \times 2 \times 4$ ’ experimental design with respect to ‘surface area’, ‘depth’, ‘predator species’ and ‘prey instar’. Nine types of glass aquarium of different sizes were used as arenas for the experiment, fully crossing three different levels of arena surface area (L $\times$ B: 10×10 , 20×20 and 30×30 cm) and three levels of water depth (5, 10 and 15 cm) (Fig. 1). Prey preferences of 24 h-starved *A. breddini* and *A. sardeus* were examined by providing 100 larvae of each of the four instars (first – fourth) of *C. quinquefasciatus* simultaneously (i.e. 400 larvae overall; $n = 5$ per experimental group). Predators were then allowed to feed for 24 h, after which they were removed. The numbers of remaining live prey within each prey group were then

counted. Controls ($n = 5$) consisted of prey in the absence of predators for all nine container treatment types.

2. 3 Prey preferences analysis

To quantify the preferences towards specific *C. quinquefasciatus* larval instar stages, Manly's selectivity index α for conditions without prey replacement was used: ^{49, 50}

$$\alpha_i = \frac{\ln((n_{i0}-r_i)/n_{i0})}{\sum_{j=1}^m \ln((n_{j0}-r_j)/n_{j0})}, i = 1, 2, \dots, m \quad (1)$$

where n_{i0} is the initial number of prey items and r_i is the number of consumed prey items by the predator of prey type i and m is the number of prey types used in the experiment. Values of α_i were then converted into electivity indices (ε_i) for displaying prey preferences: ⁵⁰

$$\varepsilon_i = \frac{m\alpha_i - 1}{(m-2)\alpha_i + 1}, i = 1, 2, \dots, m \quad (2)$$

Here, the electivity indices for individual prey types ranges from -1 (prey absent in diet) to +1 (prey representing 100% of diet), with a value of 0 corresponding to feeding without selection. Diet breadth of a predator is defined as the number of prey types with electivity index larger than -1 (i.e. all prey types consumed). Values of electivity indices for individual prey instars were compared with values expected for no electivity using separate t -tests. ^{49, 51}

Raw consumption (i.e. counts of prey killed) was analysed with respect to the 'surface area', 'depth', 'predator species' and 'prey instar' as fixed effects, and their two-way interactions, using generalised linear mixed models (GLMM) to account for repeated measures

of prey types within each experiment unit (i.e. experimental unit was a random effect as a function of prey instar). The ‘surface area $\times$ depth’ interaction term also captures density-related effects in relation to predators and prey. A negative binomial error distribution was used, owing to residual overdispersion. To derive main effects, chi-square tests (χ^2) *via* analysis of deviance with Type III sums of squares were employed. Tukey’s comparisons based on the GLMM model were used *post hoc* for pairwise comparisons of significant effects using the R package ‘emmeans’.⁵² Statistical analyses were performed in R v3.5.2.⁵³

3 RESULTS

There was 100 % survival of prey in the control groups across all container types, and thus experimental mortality was attributed to predation by notonectids. Selectivity index values, along with corresponding raw consumption counts of the mosquito instars by notonectid predators, are shown in Table S1. All four prey instars were consumed in each of the nine container sizes by both the notonectid predators (Fig. 2). Raw consumption of mosquito prey was significantly affected by container surface area and depth alone (Table 1). A significant ‘surface area $\times$ depth’ interaction term indicated emergent differences in prey consumption across gradients of water depth and surface area (Table 1). Within low water depths (Fig. 2a, b, c), consumption of prey was significantly reduced at small as compared to intermediate and large surface areas (both $P < 0.05$), but no significant consumption differences between intermediate and large surface areas were found ($P = 0.866$). Within intermediate water depths (Fig 2d, e, f), conversely, consumption of prey at high surface areas was significantly lower than small and intermediate surface areas (all $P < 0.05$), but no significant consumption between small and

intermediate surface areas was found ($P = 0.129$) (Fig. 2d, e, f). At high water depths (Fig 2g, h, i), consumption of prey was significantly higher at small as compared to intermediate surface areas ($P = 0.017$), but no significant consumptive difference between intermediate – large ($P = 0.980$) and small – large surface areas were found ($P = 0.323$).

Consumption between the two notonectid predators was significantly altered by water depth, but not surface area (Table 1). Here, *A. sardeus* consumed significantly more prey than *A. breddini* in all the three levels of water depth (all $P < 0.01$). The significant ‘predator species $\times$ prey instar’ interaction also indicated differential consumption rates for different sized prey instar between the two predators (Table 1). *Anisops sardeus* consumed significantly more third ($P < 0.001$) and fourth ($P < 0.001$) instar prey than *A. breddini*, whilst consumption of first ($P = 0.117$) and second ($P = 0.997$) instar larval prey was not significantly different between the predators (Fig. 2).

There were significant consumptive differences among mosquito prey instars by notonectids overall (Table 1). Significantly fewer fourth instar mosquito prey were consumed than all earlier instars (all $P < 0.001$), whilst second instar prey were consumed most (all $P < 0.001$). In turn, first and third instar prey consumption was not significantly different ($P = 0.306$) (Table 1). The differential consumption of prey across mosquito instar stages was significantly altered by variations in surface area and water depth (surface area $\times$ prey instar; depth $\times$ prey instar; Table 1; Fig. 2). Here, the effects of surface area were most pronounced for consumption of first and third instar mosquito larvae, with first instar prey consumed in significantly higher numbers under intermediate areas as compared to the large surface areas ($P < 0.01$), whilst third instar prey were consumed more at intermediate and large surface areas as compared to small surface areas (both $P < 0.01$). Conversely, for second and fourth instars prey, there were no

overall significant consumptive differences across each surface area (all $P > 0.05$). The significant effect of water depth among prey instar stages reflected differences only in consumption of first instar mosquito larvae, where consumption was significantly higher in low water depths as compared to intermediate depths ($P = 0.038$); low – high ($P = 0.914$) and intermediate – high depths were more similar ($P = 0.754$).

For *A. breddini*, second instar mosquito prey were positively selected irrespective of water depth or surface area variations (Fig. 3). Conversely, fourth instar prey were avoided across all container depths and surface areas. First instar *C. quinquefasciatus* prey also tended to be avoided by *A. breddini*, with avoidance strongest under high surface areas (B3, C3). Selectivity towards third instar prey was more variable according to container size (Fig. 3). Whilst third instars generally tended to be avoided, significant evidence for positive selection was evidenced at the intermediate depth under intermediate and high surface areas (B2, B3), as well as under the greatest depth at the highest surface area (C3) (Fig. 3).

Anisops sardeus also tended to positively select second instar mosquito size classes, with the exception of the shallowest depth at the lowest surface area (Fig. 4; A1), where fourth instar prey were significantly positively selected. Across all other container depths and surface areas, fourth instar prey were generally avoided. As for *A. breddini*, first instar prey tended to be avoided also, whilst there was some evidence for positive selection under the shallowest depth and highest surface area (A3), and intermediate depth with intermediate surface area (B2). Selectivity towards third instar stages was, again, highly varied (Fig. 4). Similar to *A. breddini*, positive selection towards third instars was displayed at the intermediate and high depth groups with the highest surface area treatment (B3, C3), as well as the shallow depth and intermediate

surface area group (A2). Some evidence for avoidance of third instars was displayed across other treatment groups (Fig. 4).

4 DISCUSSION

Biological control using predatory invertebrates, such as copepods, odonates and dipterans, can effectively mitigate the proliferation of medically-important mosquitoes (e.g.^{11, 16, 54, 55}). In particular, encouraging populations of predatory aquatic insects can effectively reduce larval mosquito populations in aquatic environments, and their subsequent recruitment to adult mosquito populations (e.g.^{47, 56}). Predators frequently select for prey of optimal size which they can capture with a high rate of success,^{40, 57} whilst search area (e.g. water volume) can alter foraging efficiencies.⁵⁸ The present study examines the prey preferences of two notonectids across surface area and water depth variations. In this study, it was observed that *A. sardeus* consumed significantly greater numbers of mosquito larvae than *A. breddini* across depth treatments, and better-handled late instar prey. For both predators, second instar stages of *C. quinquefasciatus* were mostly preferred, followed by third instar larvae. The largest (fourth instar) and smallest (first instar) prey sizes were generally avoided by notonectids, indicating refuge effects for small and large prey. Selectivity traits of both predators also showed emergent differences in accordance with variations in water depth and surface area. Importantly, as predator and prey numbers were fixed in each experimental unit (i.e. 1 and 400, respectively), density-related effects were observed in the interaction between surface area and water depth. This negates further consideration of predator and prey densities as variables. For both predators, avoidance of first instar prey increased in strength under greater search areas, suggesting refuge effects are exacerbated in these instances. The largest size classes of larval mosquitoes were

generally avoided irrespective of search area, suggesting recurrent handling restraints associated with larger mosquito prey. For *A. sardeus*, however, preferences towards fourth instar prey were significant under the smallest search area, further reflecting interactive complexities.

Both *A. breddini* and *A. sardeus* mostly consumed second instar prey size followed by third and first instars, whilst fourth instars were the least consumed prey size. This shows that predators were less capable of handling prey of a relatively small or large size, thereby potentially reducing population-level offtake rates *via* provisioning of refuge.^{25, 26} Consumption rates were variable according to both surface area and depth in waterbodies, indicating emergent complexities in consumption by *Anisops* according to their microhabitat. Under shallow depths, increasing surface areas generally favoured consumption, but at intermediate depth, medium sized surface areas were the most preferable container size of notonectids. At high water depth, increasing surface area had a negative impact on larval mosquito consumption. Therefore, as depth increased, notonectids were more efficacious in consuming mosquitoes under reduced surface areas. The effects of surface area and depth also manifested differently according to prey size. In particular, predation of first instar prey was significantly reduced by increases in surface area, which could enhance refugia for this prey type under specific conditions. *Anisops* species are nektonic backswimmers (see⁴⁷) that prefer to swim actively throughout the water column. Therefore, higher surface areas may impede capture efficiencies of these predators under greater depths by heightening the necessary search area to acquire prey, and particularly where prey are small-sized. Similarly, Dalal et al. (⁴⁸) showed handling times to increase with water depth, decreasing the maximum feeding rate of notonectids. Under increasing search areas this is unsurprising, as the potential for predator avoidance in mosquitoes increases given reductions in encounter rates (see⁵⁹).

Notonectidae has two sub-families: Anisopinae and Notonectinae. Anisopinae species are unique among notonectids as they can attain neutral buoyancy at water depth, and can remain within the water column for long periods of time due the presence of haemoglobin; this is unlike Notonectinae, which are positively buoyant.^{60, 61} Accordingly, it is possible that variabilities in search area manifest different among notonectid sub-families. In many ecosystems, predators prefer medium-sized prey and tend to avoid prey of relatively small or large size-classes (e.g.^{40, 57}). This indicates that prey morphology and mobility has a strong effect on the selection by predators. Attack rates and handling times are also known to be affected by the predator-prey size relationship (see⁴⁰). According to the optimal foraging theory, medium sized prey (i.e. second and third instars of mosquito larvae) would be the most profitable prey for notonectid predators.⁶² Our findings also corroborate with previous work where both the notonectids consumed mostly medium sized prey.⁴⁸ The present study thus demonstrates that irrespective of the depth and surface area variations, the choice of notonectids for a particular prey size remains largely similar. However, the effects of surface area and water depth may interact to alter overall interaction strengths. Anomalously, *A. sardeus* preferred fourth instar prey sizes under the smallest search area, which might be due to easy targeting of the highest profit prey type with minimal time searching. Nevertheless, overall, the fourth instar stage of mosquito was the most avoided prey size by both notonectid species, which is likely related to the longer handling time necessary for this prey type (see⁴⁰). This is followed by the first instar mosquito prey size (second most avoided), where predators may have had the least ‘profitability’ through energy sacrifices in the attacking and handling of this prey. A similar result has been observed in *Toxorhynchites moctezuma* (non-biting mosquito) and *Notonecta sellata* that significantly avoided first instar mosquito larvae.^{40, 63}

In conclusion, our findings reveal an overall preference for second instar prey by notonectids, while avoidance of first and fourth instar mosquito larvae was prevalent among variations in search area. Search area and water depth play an important role in predatory efficacy of aquatic organisms, where changes in depth-surface area relationships alter prey preferences and avoidance. Shifting prey preferences according to prey size by notonectid predators revealed subtle emergent effects of search area and water depth on selectivity traits. Accordingly, whilst notonectid predatory impacts are generally high, differences in water volume may result in alterations to predatory efficacy towards target species. This has implications for mosquito population management strategies targeting ephemeral aquatic ecosystems which are transient in terms of water volume. However, future work should further integrate habitat complexities with depth-surface area variations to improve quantifications of predatory impact towards pests and disease vectors.

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Table

Table 1: Analysis of variance (ANOVA) with Type III sums of squares on negative binomial generalised linear mixed model (GLMM) with respect to ‘surface area’, ‘depth’, ‘predator species’ and ‘prey instar’, and their two-way interactions. Sig. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05.

Response	Chisq	Df	Pr(>Chisq)	Sig.
(Intercept)	109.1115	1	< 2.20E-16	***
Surface area	12.6247	2	0.001814	**
Depth	11.8643	2	0.002653	**
Predator species	0.1056	1	0.74517	-
Prey instar	82.4741	3	< 2.20E-16	***
Surface area:depth	63.6286	4	5.00E-13	***
Depth:predator species	9.104	2	0.010546	*
Depth:prey instar	17.1174	6	0.008861	**
Surface area:predator species	4.0069	2	0.134868	-
Surface area:prey instar	51.0199	6	2.94E-09	***
Predator species:prey instar	45.8635	3	6.06E-10	***

Figure legends

Figure 1. Diagrammatic illustration of different sized containers used in the prey preference experiment. The three different surface areas are coded 1, 2 and 3, whilst the three different depths are A, B and C. Here, L and B are the length and breadth of the aquarium, and H is the depth of the water.

Figure 2. Mean consumption ($\pm$ SE) of four larval instars (first – fourth) of *Culex quinquefasciatus* by *Anisops breddini* (AB) and *Anisops sardeus* (AS) across water depth and search area variations. The three increasing surface areas are coded 1, 2 and 3, whilst the three increasing depths are A, B and C.

Figure 3. Electivity index values (mean $\pm$ SE) based on Manly's α for *Anisops breddini* against four instars of *Culex quinquefasciatus* across water depth and search area variations. The three increasing surface areas are coded 1, 2 and 3, whilst the three increasing depths are A, B and C. Here, positive values indicate prey preference and negative value indicate prey avoidance. The asterisk (*) mark indicates where the electivity values of the prey instars are significantly different from zero ($P < 0.05$).

Figure 4. Electivity index values (mean $\pm$ SE) based on Manly's α for *Anisops sardeus* against four instars of *Culex quinquefasciatus* across water depth and search area variations. The three increasing surface areas are coded 1, 2 and 3, whilst the three increasing depths are A, B and C. Here, positive values indicate prey preference and negative value indicate prey avoidance. The asterisk (*) mark indicates where the electivity values of the prey instars are significantly different from zero ($P < 0.05$).

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