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Accepted Version

Cuthbert, R., Callaghan, A. ORCID: https://orcid.org/0000-0002-2731-3352 and Dick, J. T. A. (2018) Dye another day: the predatory impact of cyclopoid copepods on larval mosquito Culex pipiens is unaffected by dyed environments. Journal of Vector Ecology, 43 (2). pp. 334-336. ISSN 1081-1710 doi: https://doi.org/10.1111/jvec.12318 Available at https://centaur.reading.ac.uk/76745/

To link to this article DOI: http://dx.doi.org/10.1111/jvec.12318

Publisher: Society for Vector Ecology

Scientific Note

Dye another day: the predatory impact of cyclopoid copepods on larval mosquito *Culex pipiens* is unaffected by dyed environments

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The control of vectors of parasitic diseases and arboviruses urgently requires the development of innovative measures to enhance efficacies. Cyclopoid copepods have been successful in the predatory biological control of mosquitoes, with marked *per capita* feeding rates bolstered by high abundances and suitability for container-style habitats (Marten and Reid 2007, Cuthbert et al. 2018a,b). Indeed, their application to bodies of water has resulted in the complete eradication of dengue fever from entire communities (Kay and Nam 2005). However, mosquito oviposition is highly selective in response to predators, with many mosquito species capable of detecting and avoiding predatory cues within environments fostering high densities of aquatic predators (see Vonesh and Blaustein 2010). Thus, predator cues may have strong trait-mediated consequences that may affect effective mosquito biocontrol, and so developing measures to counteract such cues is of importance for population management.

The use of pond dyes is popular in urban areas where mosquitoes proliferate, and is increasingly applied to enhance reflection and as a means of reducing the growth of algae in waterbodies (Ortiz Perea and Callaghan 2017). The application of black pond dye has been shown to be a particularly strong oviposition attractant for *Culex* mosquitoes, whereas other colored dyes have no effect (Ortiz Perea and Callaghan 2017, 2018). The strength of attraction to black dye is strong enough to reverse predator avoidance behaviors by mosquitoes (Cuthbert et al. 2018b). Thus, exploration of the use of black pond dye synergystically with predatory biocontrol agents is warranted. In particular, it is necessary to identify biocontrol agents that are not reduced in their efficacy due to pond dye effects, such as interference with the visual predation capacity of such agents. Here, we assess the impact of commercial black pond dye on the functional responses (FRs; rate of resource intake under varying densities) of two predatory cyclopoid copepods, *Macrocyclops albidus* and *Megacyclops viridis*, towards larvae of *C. pipiens*, an efficient vector of West Nile virus (Di Sabatino et al. 2014).

Macrocyclops albidus and *M. viridis* were collected from ponds within the Glastry Clay Pits, Northern Ireland (54°29'18.5"N; 5°28'19.9"W) using a polypropylene dipper. These copepods were transported in source water to Queen's University Marine Laboratory and maintained in insectary conditions (25 ± 2°C, 50-60% RH, 16:8 light:dark regime) to stimulate proliferation. Gravid females were isolated from samples with respect to each species and used to initiate pure cultures in accordance with the available literature (Marten and Reid 2007). Following first generation emergence of nauplii, originating females were dissected and identified to species. Separate copepod cultures were initiated in 10-liter tanks, and fed *ad libitum* with the protists *Paramecium caudatum* and *Chilomonas paramecium* (Sciento, Manchester, England). These protists were cultured in 2-liter flasks containing 1 liter of dechlorinated tap water and autoclaved wheat seeds *ad libitum* in insectary conditions. The prey, newly hatched *Culex pipiens* were obtained from the same laboratory where a colony had been maintained. Adult mosquitoes were kept in 32.5 × 32.5 × 32.5 cm cages and blood fed using defibrinated horse blood through a membrane feeding system (Hemotek Ltd, Accrington, England). Pads of cotton soaked in 10% sucrose solution were provided for sustenance of the mosquito colony. Egg rafts were extracted regularly from cages and were placed into 3-liter larval bowls, with hatched larvae fed *ad libitum* using ground guinea pig pellets (Pets at Home, Newtownabbey, Northern Ireland) until pupation.

To derive FRs, we supplied prey densities of 2, 4, 6, 8, 15, or 30 newly hatched 1st instar *Cx. pipiens* (1.1 – 1.3 mm) to adult female *M. albidus* or *M. viridis* (respective body lengths excluding caudal setae: 1.6 – 1.8 mm and 2.0 – 2.3 mm) over a 6 h experimental period during light conditions ($n = 4$ per experimental treatment). We starved non-ovigerous adult female copepods individually for 24 h before the experiment to standardize levels of hunger. Experiments were undertaken in arenas of 42 mm diameter containing 20 ml of dechlorinated tap water from an aerated source. Treatments either had 0.3 g/liter black liquid pond dye added, in line with the manufacturer's recommendations (Dyofix, Leeds, England), or remained undyed, in a fully factorial design, with the three factors being 'dye presence/absence,' 'predator species,' and 'prey supply level.' The source of dye was also continuously aerated to eliminate any variability in dissolved oxygen concentrations between treatments. Prey were allowed to settle for 2 h in the assigned dye treatment before the addition of predators in a fully randomized array. After 6 h, the predators were removed and the remaining prey alive were counted to derive those killed. Controls were four replicates at each prey density and dye treatment, without the presence of a predator.

All statistical analyses were undertaken in R v3.4.2. (R

Core Team 2017). We determined FR types using logistic regression of proportion of prey killed as a function of prey density. A significantly negative first order term is indicative of a Type II response, whereas a significantly positive first order term followed by a significantly negative second order term indicates a Type III response. To account for prey depletion during the experiment, we fitted the Rogers' random predator equation, integrating MLE, the maximum likelihood estimation (Bolker 2008) for conditions without prey replacement using the *frair* package in R (Pritchard et al. 2017):

$$N_e = N_0(1 - \exp(a(N_e h - T)))$$

where N_e is the number of prey eaten, N_0 is the initial density of prey, *a* is the attack constant, *h* is the handling time, and *T* is the total experimental period, in this case 6 h. The Lambert W function was applied in R to enable fitting of the random predator equation (Bolker 2008). We non-parametrically bootstrapped (n = 2,000) *a* and *h* parameter estimates to construct 95% confidence intervals (CIs) around FR curves. This process enabled the direct visual comparison of FRs through inspection of CIs (Pritchard et al. 2017).

Mosquito larvae survival in predator-free controls exceeded 99% across treatments, and thus prey deaths in predator treatments were attributed to direct predation by cyclopoids. Type II FRs were found under all treatments (Table 1), as indicated by significantly negative first-order terms. Bootstrapped (n = 2,000) 95% CIs overlapped across the entirety of the FR curves for both *M. albidus* and *M. viridis*, under both dyed and undyed treatments (Figure 1); as such, no significant difference in FR parameters (attack rate, handling time) can be assumed across all treatments among effects.

Table 1. Results of logistic regression considering prey killed as a function of prey density and parameter estimates resulting from Rogers' random predator equation.

Predator	Dye	1st order term, *p*	*a*, *p*	*h*, *p*
Ma. albidus	No	-0.05, <0.001	0.93, <0.01	0.16, <0.001
Ma. albidus	Yes	-0.06, <0.001	1.68, <0.05	0.19, <0.001
Me. viridis	No	-0.04, <0.01	0.72, <0.01	0.14, <0.01
Me. viridis	Yes	-0.06, <0.001	0.96, <0.01	0.19, <0.001

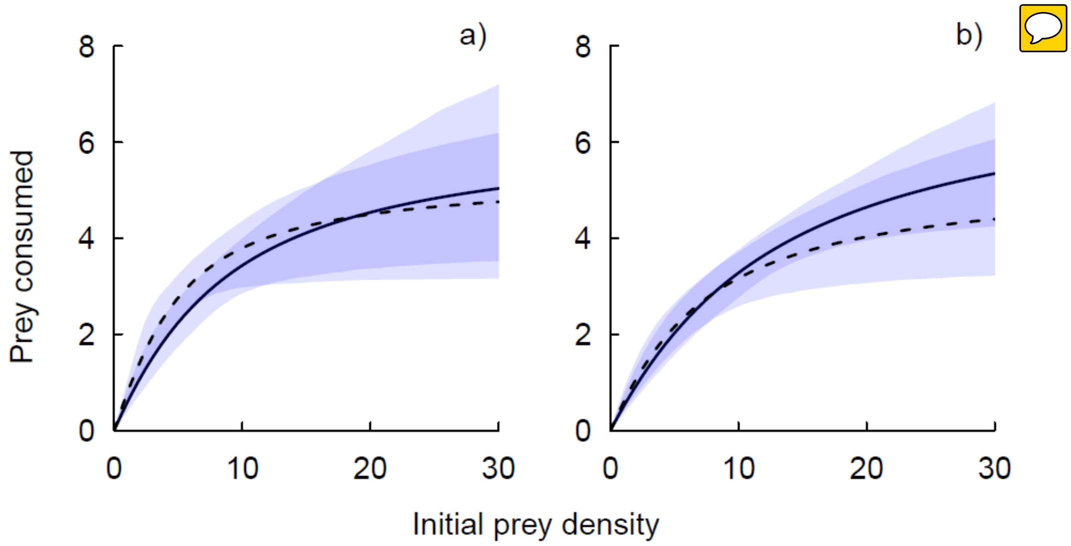

Figure 1. Functional responses (n = 4 per experimental group) of predatory copepods (a, *Macrocyclops albidus*; b, *Megacyclops viridis*) towards 1st instar *Culex pipiens*, with (dashed curve) and without (solid curve) pond dye (0.3 g/liter) over a 6 h experimental period. Shaded areas indicate bootstrapped (n = 2,000) 95% confidence intervals.

Predation by cyclopoid copepods can be effective for the control of mosquito vectors (Kay and Nam 2005) as a result of marked *per capita* predatory impacts coupled with high abundances and fecundities (Cuthbert et al. 2018a). Here, we further demonstrate strong and destabilizing predatory efficacies of two cyclopoid copepods irrespective of the addition of commercial pond dye. Such pond dyes are increasingly popular and have been demonstrated to attract mosquito oviposition (Ortiz Perea and Callaghan 2017, Cuthbert et al. 2018b). Thus, understanding the predatory impacts of such biocontrol agents in dyed environments is essential. Critically, the recurrence of Type II FRs exhibited in this study is associated with localized extinctions due to high proportional prey intake at low densities, corroborating with field efficacies of cyclopoid copepods in biocontrol. Indeed, attack rates and handling times were similar between dyed and undyed treatments. Further, previous research has demonstrated similar maximum feeding rates of cyclopoids in both simple and complex habitats (Cuthbert et al. 2018b). Our results suggest a reliance of copepods on hydromechanical rather than visual cues when foraging and are in agreement with copepod feeding results across diurnal variations reported in Hwang and Strickler (2001).

Culex mosquitoes are especially evasive of predators when ovipositing (Vonesh and Blaustein 2010), with the use of black pond dye found to profoundly attract their oviposition (Ortiz Perea and Callaghan 2017, Cuthbert et al. 2018b). Therefore, our results indicate that as predatory impacts of cyclopoid copepods are unaffected by the presence of pond dye, their use in synergy with dye may facilitate population sinks characterized by high rates of oviposition coupled with high predation rates. This may increase the vulnerability of mosquitoes to predation at the landscape level and is pertinent given that oviposition site selectivity is currently the greatest hindrance to effective larval mosquito control. We suggest further research to elucidate the effects of pond dyes on broader community interactions within aquatic ecosystems in relation to biocontrol agent selection.

Acknowledgments

This study was funded through a studentship from the Department for the Economy, Northern Ireland. *Culex pipiens* eggs were originally developed and supplied by The Pirbright Institute under BBSRC project code: BBS/E/I/00001701. We thank Maria Holyńska, Museum and Institute of Zoology, Warsaw, Poland, for assistance with copepod identification.

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