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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 had the most success in the predatory biological control of vectorially important 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 waterbodies has resulted in the complete eradication of dengue fever from entire communities (e.g. Kay and Nam 2005). However, oviposition by mosquitoes 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 which may impact 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

24 waterbodies (Ortiz-Perea and Callaghan 2017). The application of black pond dye has been
25 shown to be a particularly strong oviposition attractant for *Culex* mosquitoes, whereas other
26 coloured dyes have no effect (Ortiz-Perea and Callaghan 2017, Ortiz-Perea et al. 2018). The
27 strength of attraction to black dye is strong enough to reverse predator avoidance behaviours
28 by mosquitoes (Cuthbert et al. 2018b). Thus, exploration of the use of black pond dye
29 synergistically with predatory biocontrol agents is warranted. In particular, it is necessary to
30 identify biocontrol agents that are not reduced in their efficacy due to pond dye effects, such
31 as interference with the visual predation capacity of such agents. Here, we assess the impact
32 of commercial black pond dye on the functional responses (FRs; rate of resource intake under
33 varying densities) of two predatory cyclopoid copepods, *Macrocyclus albidus* and
34 *Megacyclus viridis*, towards larvae of *C. pipiens*, an efficient vector of West Nile virus (e.g.
35 Di Sabatino et al. 2014).

36 *Macrocyclus albidus* and *M. viridis* were collected from ponds within the Glastry
37 Clay Pits, Northern Ireland (54°29'18.5"N 5°28'19.9"W) using a polypropylene dipper. These
38 copepods were transported in source water to Queen's University Marine Laboratory, and
39 maintained in insectary conditions (25 ±2 °C, 50 – 60% RH, 16:8 light:dark regime) to
40 stimulate proliferation. Gravid females were isolated from samples with respect to each
41 species and used to initiate pure cultures in accordance with the available literature (Marten
42 and Reid 2007). Following first generation nauplii emergence, originating females were
43 dissected and identified to species. Separate copepod cultures were initiated in 10 L tanks,
44 and fed ad libitum with the protists *Paramecium caudatum* and *Chilomonas paramecium*
45 (Sciento, Manchester, England). These protists were cultured in 2 L flasks containing 1 L
46 dechlorinated tap water and autoclaved wheat seeds *ad libitum* in insectary conditions. The
47 prey, newly hatched *C. pipiens*, were obtained from the same laboratory where a colony had
48 been maintained. Adult mosquitoes were kept in 32.5 × 32.5 × 32.5 cm cages (Bugdorm,

49 Watkins and Doncaster, Leominster, England) and blood fed using defibrinated horse blood
50 (TCS Biosciences, Buckingham, England) through a membrane feeding system (Hemotek
51 Ltd, Accrington, England). Pads of cotton soaked in 10% sucrose solution were provided for
52 sustenance of the mosquito colony. Egg rafts were extracted regularly from cages and were
53 placed into 3 L larval bowls, with hatched larvae fed *ad libitum* using ground guinea pig
54 pellets (Pets at Home, Newtownabbey, Northern Ireland) until pupation.

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

70 All statistical analyses were undertaken in R v3.4.2. (R Core Team 2017). We
71 determined FR types using logistic regression of proportion of prey killed as a function of
72 prey density. A significantly negative first order term is indicative of a Type II response,
73 whereas a significantly positive first order term followed by a significantly negative second

74 order term indicates a Type III response (Juliano 2001). To account for prey depletion during
75 the experiment, we fitted the Rogers' random predator equation, integrating maximum
76 likelihood estimation (MLE, Bolker 2010), for conditions without prey replacement using the
77 *frair* package in R (Juliano 2001; Pritchard et al. 2017):

$$78 \quad N_e = N_0(1 - \exp(a(N_e h - T)))$$

79 Eqn. 1

80 where N_e is the number of prey eaten, N_0 is the initial density of prey, a is the attack constant,
81 h is the handling time and T is the total experimental period, in this case 6 h. The Lambert W
82 function was applied in R to enable fitting of the random predator equation (Bolker 2008).
83 We non-parametrically bootstrapped ($n = 2000$) a and h parameter estimates to construct 95%
84 confidence intervals (CIs) around FR curves. This process enables the direct visual
85 comparison of FRs through inspection of CIs (see Pritchard et al. 2017).

86 Mosquito larvae survival in predator-free controls exceeded 99% across treatments,
87 and thus prey deaths in predator treatments were attributed to direct predation by cyclopoids.
88 Type II FRs were found under all treatments (Table 1), as indicated by significantly negative
89 first order terms. Bootstrapped ($n = 2000$) 95% CIs overlapped across the entirety of the FR
90 curves for both *M. albidus* and *M. viridis*, under both dyed and undyed treatments (Figure 1);
91 as such, no significant difference in FR parameters (attack rate, handling time) can be
92 assumed across all treatments among effects.

93 Predation by cyclopoid copepods can be effective in the control of disease vector
94 mosquitoes (e.g. Kay and Nam, 2005) as a result of marked *per capita* predatory impacts
95 coupled with high abundances and fecundities (Cuthbert et al. 2018a). Here, we further
96 demonstrate strong and destabilising predatory efficacies of two cyclopoid copepods
97 irrespective of the addition of commercial pond dye. Such pond dyes are increasingly popular

98 and have been demonstrated to attract mosquito oviposition (Ortiz-Perea and Callaghan 2017;
99 Cuthbert et al. 2018b). Thus, understanding the predatory impacts of such biocontrol agents
100 in dyed environments is essential. Critically, the recurrence of Type II FRs exhibited in this
101 study is associated with localised extinctions due to high proportional prey intake at low
102 densities, corroborating with field efficacies of cyclopoid copepods in biocontrol. Indeed,
103 attack rates and handling times were similar between dyed and undyed treatments. Further,
104 previous research has demonstrated similar maximum feeding rates of cyclopoids in both
105 simple and complex habitats (Cuthbert et al. 2018b). Our results here suggest a reliance of
106 copepods on hydromechanical rather than visual cues when foraging, and are in agreement
107 with copepod feeding results across diurnal variations reported in Hwang and Strickler
108 (2001).

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

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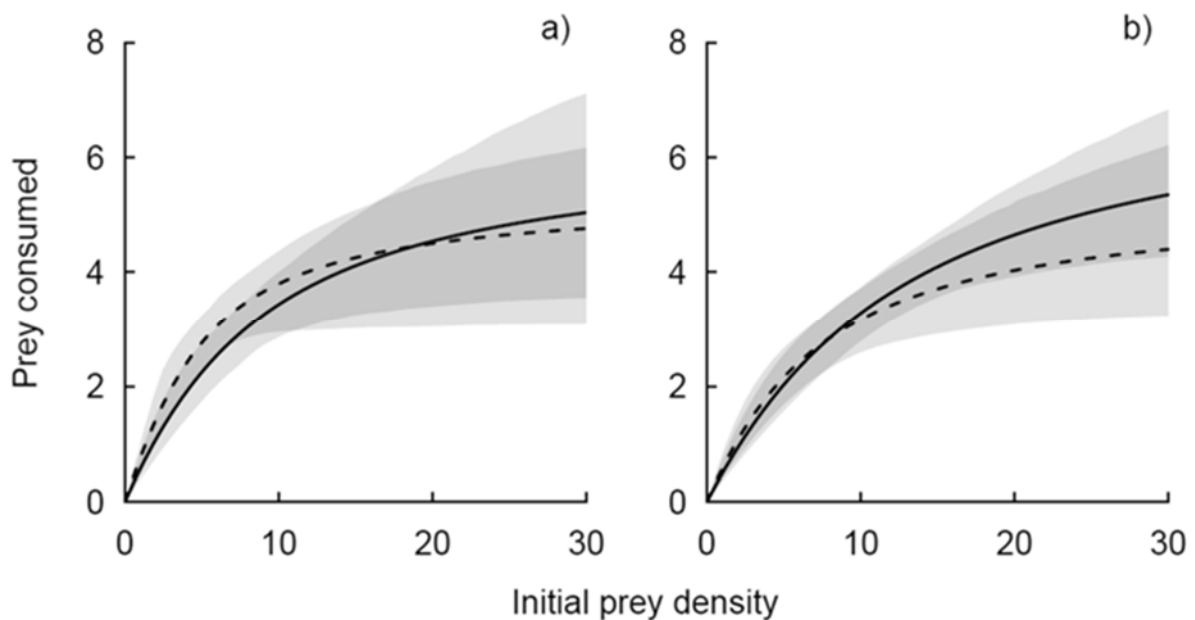
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167 Table 1. Results of logistic regression considering prey killed as a function of prey density,
 168 and parameter estimates resulting from Rogers' random predator equation.

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

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171 Figure 1. Functional responses ($n = 4$ per experimental group) of predatory copepods (a,
 172 *Macrocyclus albidus*; b, *Megacyclus viridis*) towards first instar *Culex pipiens*, with
 173 (dashed curve) and without (solid curve) pond dye (0.3 g L^{-1}) over a 6 hour experimental
 174 period. Shaded areas indicate bootstrapped ($n = 2000$) 95% confidence intervals.

175