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1 Foraging Efficacy of Non-native Crayfish Under Nitrate Pollution

2
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10 11 **Abstract**

12 Life history traits such as predation and foraging are key behavioural mechanisms through
13 which invasive, non-native species can have a detrimental effect on the resident flora and fauna
14 of recipient ecosystems. Environmental change may alter these traits in ways that are currently
15 poorly understood. In aquatic ecosystems, changes to the environment such as nitrate pollution
16 can have chronic, unforeseen consequences for both native and invasive species. As keystone
17 species and ecosystem engineers, non-native crayfish species can be highly destructive through
18 predating on native species from multiple trophic levels. Here, we assessed the effect of nitrate
19 concentration on the foraging behaviour of two invasive crayfish species from the UK
20 (*Pacifastacus leniusculus* and *Orconectes virilis*), particularly focussing on whether there were
21 any species-specific effects of nitrate. During experimental trials, elevated nitrate concentration
22 had a similar impact on the foraging efficacy of both *P. leniusculus* and *O. virilis*, slowing both
23 their initial reaction and latency to feed. Nitrate also reduced the ability of both crayfish species
24 to catch live prey as well as the total number of prey consumed. Though both species were
25 similarly influenced by nitrate, we highlight that even at levels deemed safe for drinking water
26 (50 mg NO₃⁻/L), nitrate concentration can significantly impact the behavioural traits of aquatic
27 species. In the case of invasive species, fluctuations in environmental nitrate concentration
28 could therefore have implications for invader success and impacts on the wider ecosystem.

29
30 **Keywords:** eutrophication; nitrate; foraging; invasive non-native species (INNS); behavioural
31 traits; Water Framework Directive

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40 Introduction

41 Invasive, non-native species are a leading cause of biodiversity loss worldwide (Mack et al.
42 2000; Sala et al. 2000), but only a small percentage of non-native species that are introduced
43 subsequently become invasive (Williamson and Fitter 1996). The success of introduced species
44 is often associated with particular life history traits, such as high reproductive output, disease
45 resistance and/or tolerance to pollution (Colautti et al. 2004; Maceda-Veiga et al. 2013;
46 Vilcinskas et al. 2015), as well as behavioural traits such as aggression and foraging (Holway
47 and Suarez 1999; Viana et al. 2016). Aggressive, voracious invaders are often competitively
48 dominant over other species when competing for resources (Vorburger and Ribic 1999; Nakata
49 and Goshima 2003; Strayer 2010). However, traits associated with invasive animals may
50 change due to altered environmental conditions and increasing pressure from anthropogenic
51 disturbances (Guan 1994; Holway and Suarez 1999; Wong and Candolin 2015). Ultimately,
52 this could result in invaders losing their competitive advantage under differing environmental
53 regimes and it is important to understand how predicted future global changes may influence
54 the traits that make non-native species successful.

55 Nutrient pollution is one of the most pernicious forms of global change in aquatic
56 ecosystems, resulting in cultural eutrophication (Camargo and Alonso 2006; Smith and
57 Schindler 2009). Increased levels of inorganic nitrogen such as ammonia (NH_3), nitrite (NO_2^-)
58 and nitrate (NO_3^-) can enhance ecosystem productivity, but can also have devastating effects
59 on freshwater organisms (Camargo and Alonso 2006; Hickey and Martin 2009). The toxicity
60 of nitrate is poorly studied compared to ammonia and nitrite, but growing evidence indicates
61 that chronic nitrate exposure can profoundly alter the life-history traits of fish (Smallbone et
62 al. 2006; Scott and Sloman 2009), amphibians and invertebrates (Maceda-Veiga et al. 2015;
63 Guilette and Edwards 2005; Camargo et al. 2006). Behavioural studies can link physiological
64 and ecological processes and may identify the subtle effects of chemical toxicity in aquatic
65 ecosystems (Scott and Sloman 2004). One of the best indicators of toxic effects on aquatic
66 organisms is reduced foraging efficiency (Colin et al. 2016), as reported in *Daphnia magna*
67 under chronic nitrate exposure (Maceda-Veiga et al. 2015). Pollutants can alter the perception
68 of visual and chemical cues (Halfwerk and Slabbekoorn 2015), with ramifications for the
69 detection of food but also conspecifics and predators (Troyer and Turner 2015). However, the
70 effect of nitrate on the sensory capacity of aquatic organisms has not yet been investigated (but
71 see Camargo et al. 2005), and it is of primary interest considering nitrate levels are expected to
72 increase (Galloway et al. 2008).

73 Invasion of freshwater ecosystems is non-random both in terms of taxon and the
74 biological traits of invaders, with efficient predatory decapods, molluscs and fish being
75 particularly prevalent (Strayer 2010). Crayfish are extremely successful decapod invaders,
76 which are keystone species and ecosystem engineers that have a significant impact on
77 freshwater ecosystems through profoundly modifying their habitat and influencing all levels of
78 the food web through predation and herbivory (see Holdich et al. 2009; Williams et al. 2010;
79 James et al. 2014). Despite this, research on the effects of nitrate enrichment on invasive
80 crayfish performance is limited. The survival, feeding and escape response of the threatened
81 European white-clawed crayfish (*Austropotamobius italicus*) was negatively affected by
82 ecologically relevant nitrate concentrations (Benítez-Mora et al. 2014). In contrast, the
83 presence of invasive red-swamp crayfish (*Procambarus clarkii*) seems to be related to nutrient
84 enriched waters in Mediterranean rivers (Maceda-Veiga et al. 2013). Nonetheless, there is a
85 distinct lack of experimental or observational evidence of nitrate toxicity to other crayfish
86 species living in nitrate vulnerable zones.

87 The current experimental study tested for the effects of ecologically relevant nitrate
88 concentrations on feeding efficacy of two invasive crayfish, the signal (*Pacifastacus*
89 *leniusculus*) and virile (*Orconectes virilis*) crayfish in the River Lea, London, UK. The virile

90 crayfish was introduced comparatively recently (first detected in 2004), whilst the signal
91 crayfish has been established in this catchment for over three decades (Ahern et al. 2008).
92 Virile crayfish are competitively dominant over signal crayfish, which is leading to an apparent
93 displacement of the signal crayfish (James et al. 2015) in this designated nitrate vulnerable
94 zone (Environment Agency 2015). However, this competitive advantage may be altered
95 depending on nitrate conditions. As crayfish feed on benthic invertebrates with key roles in
96 ecosystem processes (e.g. filter feeders, shredders; Graça 2001), alterations in the number and
97 type of prey ingested due to nitrate may influence predator metabolism with ramifications for
98 ecosystem function.

99

100 **Materials and Methods**

101 *Collection of study animals*

102 Signal (*Pacifastacus leniusculus*) and virile (*Orconectes virilis*) crayfish were trapped from
103 allopatric sites in the River Lee, London, UK (Signal crayfish - NGR TL 38414 07906; Virile
104 crayfish - NGR: TL 36834 02891) during October 2014 using standardised trapping protocols
105 ('trappy traps' baited with tinned fish and checked daily for two days). Between 2008 and
106 2012, data obtained from the Environment Agency during water quality monitoring on the
107 River Lee at a site within 10 km of the trapping points, showed that nitrate concentration ranged
108 between 33 and 72 mg NO₃⁻/l, with an average of 46.4 mg NO₃⁻/l. Water samples were collected
109 monthly, and nitrate concentration determined using a standard method (Standing Committee
110 of Analysts, 1987). Crayfish were subsequently transported back to Cardiff University in
111 plastic tanks with source water and air pumps.

112

113 *Animal maintenance*

114 Upon arrival in the lab, crayfish were housed individually in 15 L tanks containing either <10
115 or 50mg NO₃⁻/L under a 16h:8h light/dark regime at 14±1°C for 14 days. Each holding tank
116 contained gravel substrate (2 cm) and a refuge (plastic pot). The two nitrate concentrations
117 represented clean dechlorinated tap water (<10mg NO₃⁻/L), and the higher nitrate level
118 corresponds to the safe threshold for nitrate concentrations for drinking water in Europe (50
119 mg NO₃⁻/L; Council of the European Union 1998). Nitrate solutions were hand-made using
120 potassium nitrate (KNO₃) as a nitrate source before we fully renewed water in each container
121 every other day. To ensure the experimental conditions remained stable throughout the
122 experiment, chemical water properties were measured using Sera® colourimetric test kits
123 (previously used in our laboratory, Maceda-Veiga *et al.*, 2015; Smallbone *et al.*, 2016), where
124 pH=7, carbonate water hardness (dKH) = 5, [ammonia] < 0.5 mg L⁻¹, [nitrite] < 0.5 mg L⁻¹ and
125 [nitrate] < 10 or 50 mg L⁻¹). All animals were measured (range 38-67 mm carapace length) and
126 sexed. Any crayfish with missing chela were omitted from the study. Crayfish were fed every
127 other day with Tetra Crusta crayfish food pellets and frozen peas 20 min prior to the water
128 change to maintain precise nitrate concentrations. Upon termination of experiments, all animals
129 were humanely destroyed by freezing at -20°C, as under Section 14 of the Wildlife and
130 Countryside Act 1981 it is prohibited to release or maintain invasive species in the long term.

131

132 *Foraging behaviour*

133 The foraging behaviour of individual signal and virile crayfish were assessed over two days
134 after the fortnight acclimatisation period, under the same nitrate concentrations: <10 and 50mg
135 NO₃⁻/L (bloodworm trials: N = 17, 27; gammarid trials N = 17, 31). Crayfish were individually
136 transferred to one end of an experimental arena (L60 cm x W30 cm x D30 cm) and foraging
137 trials were repeated using different prey types, either; 5 live *Gammarus pulex* (referred to as
138 gammarids) or 20 defrosted *Tubifex* worms (referred to as bloodworm), with half of the
139 crayfish being fed gammarids and half being fed bloodworm first, and then fed the alternative

140 prey item on the second day. Both crayfish and prey were initially restrained within opaque
141 glass cylinders that were simultaneously lifted at the start of the experiment, thus ensuring that
142 crayfish were equidistant from the prey items across trials. For each trial we recorded; latency
143 to feed (first reaction), time to first prey capture, and total number of prey consumed within 30
144 min. Crayfish which did not feed on bloodworm within the 30 min observation period were
145 recorded as non-responders and excluded from further analysis. In this study, gammarids were
146 used as an example of a live prey item; however, since many crayfish failed to catch gammarids
147 during the trials, these data were converted into a ‘fed/did not feed’ response.

148

149 *Statistical analyses*

150 All statistical analyses were performed using R statistical software (R Core Team, 2012).
151 General Linear Models (GLMs) were used to determine the effects of nitrate concentration on
152 feeding responses. Tukey’s HSD post-hoc comparisons of significant results were then applied
153 for pairwise comparisons, using the ‘lsmeans package’ (Lenth 2015). All models also included
154 crayfish sex, species and carapace length, as well as an interaction term between nitrate and
155 species to investigate species-specific effects of nitrate concentration (Table 1). A large number
156 of crayfish (N = 34) failed to feed on gammarids in 30 min, leading to a low sample size,
157 therefore the data were converted to a yes/no feeding response variable (i.e. whether the
158 crayfish fed on gammarids in 30 min or not). Error family and link functions (Table 1) were
159 chosen to give the best fit and meet model assumptions (Crawley, 2007) and models were
160 refined by stepwise deletion, removing the least significant term and re-running the model until
161 only significant (P<0.05) terms remained (Crawley, 2007). Residual diagnostic plots from the
162 models were used to verify the assumptions of normality and homoscedasticity (Zuur et al.
163 2010).

164

165 **Results**

166 At 50 mg NO₃⁻/L, both signal and virile crayfish were slower to initially react to bloodworm
167 than conspecifics at <10 mg NO₃⁻/L (Tukey HSD, P=0.019; Fig. 1). Overall, virile crayfish
168 were slower to react to bloodworm than signal crayfish (Tukey HSD, P=0.009; Table 1; Fig.
169 1).

170

171 **Table 1** - Model output from GLM (General Linear Model) stepwise refinement and post-
172 hoc pairwise comparisons (TukeyHSD) of crayfish reaction.

Dependent variable	Prey item	Model family, link function	Fixed terms	F value [§]	df	p-value	Post-hoc contrasts	p-value	Estimate
Time to first reaction	Bloodworm*	Gaussian, identity	Species	6.904	1, 41	0.012	Signal v. virile <10 v. 50	0.009 0.019	-0.806 -0.740
			Nitrate	5.512	1, 41	0.024			
			Sex	0.622	1, 40	0.435			
			CL	0.120	1, 38	0.731			
			Species:Nitrate	0.228	1, 39	0.636			
	Gammarids*	Gaussian, identity	Species	0.036	1, 43	0.852			
			Nitrate	3.196	1, 46	0.081			
			Sex	1.429	1, 44	0.238			
			CL	1.935	1, 45	0.171			
			Species:Nitrate	0.069	1, 42	0.794			
Time to first feed	Bloodworm**	Gamma, identity	Species	0.849	1, 41	0.362	<10 v. 50	<0.001	-6.885
			Nitrate	11.201	1, 42	0.002			
			Sex	4.044	1, 39	0.494			
			CL	0.522	1, 38	0.474			
			Species:Nitrate	3.889	1, 40	0.056			
Fed or not	Gammarids	Binomial, cloglog	Species	0.003	1, 43	0.955	<10 v. 50	0.009	1.145
			Nitrate	6.626	1, 46	0.010			
			Sex	1.626	1, 44	0.202			
			CL	3.748	1, 45	0.053			
			Species:Nitrate	-	-	-			
Prey consumed	Bloodworm	Quasipoisson, sqrt	Species	1.274	1, 41	0.266			
			Nitrate	1.080	1, 40	0.305			
			Sex	0.560	1, 38	0.459			
			CL	0.428	1, 39	0.517			
			Species:Nitrate	0.699	1, 40	0.408			
	Gammarids	Quasipoisson, sqrt (+1)	Species	12.365	1, 44	0.001	Signal v. virile <10 v. 50	<0.001 <0.001	0.293 0.418
			Nitrate	15.627	1, 44	<0.001			
			Sex	1.393	1, 43	0.244			
			CL	8.353	1, 44	0.006			
			Species:Nitrate	1.211	1, 42	0.278			

*log transformed
**squareroot transformed

[§]LRT for binomial model

174 **Fig. 1 – Nitrate concentration affects the initial reaction of crayfish to food.** Initial reaction
175 (s) of signal (*Pacifastacus leniusculus*; circles) and virile (*Orconectes virilis*; squares) crayfish
176 to bloodworm prey at <10 and 50 mg NO₃⁻/L (log transformed). Points with shared letters
177 denote non-significant differences (P > 0.05)

178

179 For both crayfish species, the time taken for them to subsequently feed on bloodworm was
180 also significantly slower at 50 mg NO₃⁻/L (Tukey HSD, P=0.001; Fig. 2).

181

182 **Fig. 2 – Nitrate concentration influences the feeding response of crayfish.** First feeding
183 reaction (s) of signal (*Pacifastacus leniusculus*; circles) and virile (*Orconectes virilis*; squares)
184 crayfish to bloodworm at <10 and 50 mg NO₃⁻/L (square-root transformed). Points with shared
185 letters denote non-significant differences (P > 0.05)

186

187 The total number of bloodworm eaten was not influenced by nitrate concentration, crayfish
188 species, carapace length nor sex.

189 The initial reaction of crayfish to gammarids was not significantly influenced by nitrate,
190 sex, species or carapace length of crayfish (Table 1). Overall however, crayfish were less likely
191 to catch and feed on gammarids at 50 mg NO₃⁻/L compared to <10 mg NO₃⁻/L ($t_{1,46} = -2.535$,
192 $P = 0.015$). The total number of gammarids eaten was also significantly influenced by nitrate
193 concentration, species (Fig. 3) and carapace length (Table 1). Crayfish at 50 mg NO₃⁻/L ate
194 fewer gammarids than those at <10 mg NO₃⁻/L (Tukey HSD, P<0.001), virile crayfish ate less
195 than virile crayfish (Tukey HSD, P<0.001), and larger crayfish ate fewer gammarids overall.

196

197 **Fig. 3 – Number of live gammarid prey consumed.** Number of gammarids consumed by
198 signal (*Pacifastacus leniusculus*) and virile (*Orconectes virilis*) crayfish at <10 and 50 mg
199 NO₃⁻/L. Points with shared letters denote non-significant differences (P > 0.05)

200

201 Discussion

202 This experimental study has shown that nitrate, even at levels deemed safe for use as drinking
203 water (Council of the European Union 1998), can reduce the foraging efficacy of aquatic non-
204 native species in the UK. The effects of high nitrate are generally not lethal to crayfish; in fact,
205 they appear to be relatively tolerant compared to other aquatic organisms (Jensen 1996;
206 Benítez-Mora et al. 2014). However, the current study has shown that elevated nitrate may alter
207 the predatory impact of crayfish on other organisms, a key trait that makes non-native crayfish
208 particularly troublesome invaders. Altered behavioural responses that affect consumption can
209 significantly alter food web structure (Wong and Candolin 2015), which may be particularly
210 true in terms of keystone, invasive species that exert a disproportionately high impact on
211 ecosystems.

212 Previous studies have shown that the chemical environment influences the perception
213 and sensory performance of aquatic organisms (Troyer and Turner 2015; Halfwerk and
214 Slabbekoorn 2015). In the current study, we show that elevated nitrate concentration slows the
215 reaction time and foraging efficacy of non-native crayfish species. A previous study Benítez-
216 Mora et al. (2014) showed a similar effect of nitrate on native European crayfish (*A. pallipes*)
217 foraging efficacy. Crayfish have chemosensory hairs within their ambulatory feet (Fedotov
218 2009) and slower reactions to prey at higher nitrate concentrations suggests that nitrate can
219 interfere with the sensitivity of crayfish to chemical cues from prey. Although the behaviour
220 of the gammarids themselves in the current study may have been directly affected by elevated
221 nitrate, a previous study suggested that nitrate concentrations of up to 128 mg NO₃⁻/L had little
222 effect on *Gammarus pseudolimnecus* (see Stelzer and Joachim 2010).

223 Crayfish have wide-ranging impacts on ecosystems, largely through their omnivorous
224 foraging nature (Lodge et al. 2000; Geiger et al. 2005; Bobeldyk and Lamberti 2008; Jackson
225 et al. 2014) and a reduction in their foraging efficacy may alter the impacts of crayfish in high-
226 nitrate areas. The two non-native species of crayfish in the present study, the signal and virile
227 crayfish, are of particular interest in the UK due to the widespread invasion by signal crayfish
228 since the 1980s, the recent introduction and continued invasion of the virile crayfish (Ahern et
229 al. 2008), and the potential competitive dominance of the virile crayfish, apparently displacing
230 the established signal crayfish in the River Lea catchment (James et al. 2015). Whilst the virile
231 crayfish reacted slower to bloodworm and ate fewer gammarids than signal crayfish, both
232 species were similarly affected by elevated nitrate, suggesting that nitrate conditions are
233 unlikely to alter the competitive relationship between these two species. A key finding
234 however, is that both crayfish may be less successful at foraging in high-nitrate areas than those
235 present in low-nitrate areas.

236 The current study has shown that elevated nitrate at ecologically relevant levels can
237 lead to significant changes to behavioural traits associated with invasive species in aquatic
238 environments. Sub-optimal foraging conditions for non-native species that are introduced to
239 new, high-nitrate environments may have implications for the “three-tens” rule of
240 establishment of a non-native species (Williamson and Fitter 1996), where high-nitrate areas
241 could be slightly more ‘resistant’ to invasion. In this case, aquatic non-native species may be
242 at a relative disadvantage in disturbed, urban areas of high-nitrate, and may be even more
243 successful when introduced to ecosystems less affected by excess nitrate.

244

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