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1 **Comparative feeding rates of native and invasive ascidians**

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3 Tedi Hoxha^{1*}, Steve Crookes^{1,2*}, Christophe Lejeune³, Jaimie T.A. Dick⁴, Xuexiu Chang⁵,
4 Sarah Bouchemousse^{3,6}, Ross N. Cuthbert⁴ and Hugh J. MacIsaac^{1,5}

5
6 ¹ Great Lakes Institute for Environmental Research, University of Windsor, Windsor, Ontario,
7 N9B 3P4, Canada

8 ² Biodiversity Institute of Ontario, University of Guelph, Guelph, Ontario, N1G 2W1, Canada

9 ³ Sorbonne Université, CNRS, UMR 7144 AD2M, Station Biologique de Roscoff, Place Georges
10 Teissier, 29680 Roscoff, France

11 ⁴ Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast,
12 Belfast, Northern Ireland, BT9 7BL, UK

13 ⁵ School of Ecology and Environmental Sciences, Yunnan University, Kunming 650091, China

14 ⁶ Department of Biology, University of Fribourg, Chemin du musée 10, 1700 Fribourg,
15 Switzerland

16
17 * Authors contributed equally

18
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21 **Correspondence:** hughm@uwindsor.ca, hughm@uwindsor.ca; ph. (+1)519 253-3000 ext. 3754,
22 Fax (+1) 519 971-3616

23

24 **Abstract**

25 Ascidians have a recent history of species introductions globally, often with strong ecological
26 impacts. Comparisons of per capita effects of invaders and comparable natives are useful to
27 assess such impacts. Here, we explore ingestion rates (IR) and clearance rates (CR) of *Ciona*
28 *intestinalis* and *Ciona robusta*, co-occurring native and non-native ascidians, respectively, from
29 Brittany, France. IR was positively related to food concentration, with the invader responding
30 more strongly to increasing food concentration. CR also differed by species, with the invader
31 demonstrating higher values. *C. robusta* exhibited a higher functional response (Type I) than did
32 *C. intestinalis* (Type II). Relative impact measured using seasonal abundance and IR revealed
33 that *C. robusta* has a much greater impact than *C. intestinalis* at all food concentrations tested,
34 though the former has a constrained distribution which limits its regional impact. Nevertheless,
35 when abundant, we expect *C. robusta* to exert a greater impact on algal foods.

36

37 **1. Introduction**

38 Aquatic invasive species (AIS) are increasingly common in both marine and freshwater
39 habitats world-wide owing to a combination of intentional (e.g. stocking) and unintentional (e.g.
40 hull fouling, ballast water) introductions (e.g. Ruiz et al. 2000; Ricciardi 2006). AIS are among
41 the strongest stressors in many aquatic ecosystems (e.g. Clavero and Garcia-Berthou 2005; Allen
42 et al. 2013; Arthington et al. 2016), with a subset of introduced species exerting strong ecological,
43 health and/or economic costs.

44 Ascidians are primarily sessile organisms with a brief pelagic (lecithotrophic) larval stage.
45 Numerous ascidian species have experienced an increase in global range linked to human-
46 mediated spread and, in some cases, climate warming (e.g. Lambert 2001, 2007; Sorte et al.

47 2010; Bock et al. 2011; Zhan et al. 2015; Simkanin et al. 2016; Nydam et al. 2017). In total,
48 Zhan et al. (2015) catalogued 80 species that were recognized as non-native in the habitats in
49 which they were reported. In the Netherlands, non-native ascidians colonized during two major
50 spates, one in 1974-1977, the other in 1991-2004 (Gittenberger 2007). The increase in the
51 number of non-native ascidian species reported in the USA was low but relatively linear between
52 1850 and 1950, thereafter increasing sharply on both Pacific and Atlantic coasts (Simkanin et al.
53 2016).

54 On the English Channel coast of Brittany, France, non-native *Ciona robusta* (previously
55 *Ciona intestinalis* type A) co-occurs with *C. intestinalis* (previously *C. intestinalis* type B)
56 (Brunetti et al. 2015; Bouchemousse et al. 2016a). While the arrival date of *C. robusta* is not
57 known, it is believed to have established since the turn of the 21st century (Bouchemousse et al.
58 2016a). As the species occupy the same habitats and are filter feeders, they have the potential to
59 compete for settling substrates or for food (Bouchemousse et al. 2017).

60 Human-mediated range enhancement of ascidians has been effected mainly by fouling on
61 ships, transfer on equipment or as a fellow traveler on aquaculture stock, and by fishery or
62 recreational boats, although there is a small likelihood of transfer in ballast water (see Zhan et al.
63 2015). As introduced ascidians often adversely affect recipient communities, their spread is
64 cause for concern (see Lambert 2009; Zhan et al. 2015).

65 Identifying which introduced species are likely to produce strong impacts is a daunting
66 challenge owing to the varying nature of the species themselves, the nature of the ecosystems
67 that they are introduced into, and a variety of context-dependencies (Kumschick et al. 2015).
68 Recently, Dick et al. (2014) proposed analyzing comparative functional responses (FR) of
69 introduced species (or those that might be introduced) versus those of comparable native taxa to

70 assess whether the former would have high impact. The functional response considers a species'
71 *per capita* resource consumption as a function of resource availability, and Dick et al.'s (2014)
72 study highlighted that invader FRs typically exceed those of native species. This approach was
73 then extended by combining it with species abundance data to yield a total impact potential for
74 the invader scaled to that of the native species (Dick et al. 2017a).

75 In this study, we explore the comparative feeding ecology of non-native *Ciona robusta*
76 and native *C. intestinalis* to determine whether these taxa have similar feeding attributes and
77 expected ecological effects, or whether the general pattern of invader > native that Dick et al.
78 (2014) identified also holds for these very similar ascidians.

79 2. Methodology

80 2.1 Sampling procedure

81 *Ciona* individuals were collected by scraping from pontoons and pillars in Brest, France
82 on 25th September, 2015 and acclimated in the animal husbandry lab (18°C) at the Roscoff
83 Biological Station, France. *Ciona* cultures were maintained on *Isochrysis affinis galbana* algae
84 (issued from the Roscoff Culture Collection (RCC) facility under the reference numbers
85 RCC1349) at concentrations of 15-20 x10⁶ cells/mL. Seven experimental food concentration
86 treatments (1508, 3380, 5900, 12873, 29539, 51616, 133084 cells/mL) were determined using a
87 Malassez cell counting chamber. Experimental *Ciona* individuals were housed in separate
88 cylindrical tanks, each filled with 2 L seawater, totaling seven tanks. Six of the seven tanks
89 contained *Ciona* individuals, with the remaining one containing only *Tisochrysis*, thereby
90 serving as a control. Of the six experimental tanks, three contained a single *C. intestinalis*
91 individual and food, while the other three had a single *C. robusta* individual and food. *Ciona*
92 individuals were randomly selected and distinguished morphologically. This design was repeated

93 twice to produce six replicates for each ascidian species at each algal concentration, with the
94 exception of the treatment with 12783 algal cells/mL, where time constraints only allowed for
95 three replicates of each species.

96 2.2 Experimental setup

97 At the beginning of each trial, individuals were placed upright at the midpoint of each
98 tank, while 10 mL of concentrated algae suspension was injected into the centre of the tank. The
99 algal suspension was mixed using an air bubbler affixed with plasticine to the side of each tank.
100 Feeding trials were run for 1 hr, after which algal cell counts were obtained using flow cytometry,
101 focusing on cells 3.5-6 μm in diameter (Bendif et al. 2013). Three replicate 1.5 mL samples were
102 collected from the centre of each tank (2 cm below the surface) to assess algal concentration, and
103 stored in 1.6 mL Eppendorf tubes containing 15 μL of 25% glutaraldehyde at -80°C for
104 preservation. Cell densities were then quantified using a Cell Lab Quanta Flow Cytometer
105 (Beckman Coulter, Inc.) at a calibrated flow rate of 30 $\mu\text{L min}^{-1}$, and cell counts were converted
106 to cells/mL. Final and initial algal concentrations in experimental tanks were compared with a
107 correction for controls lacking animals. Following the experiment, the middle gut (from the
108 stomach to the anus) of each individual was excised using 10% bleach-sterilized razor blades to
109 ensure the mass of ingested food was excluded from subsequent body mass measurements.
110 Individuals were then desiccated in an oven at 65°C to obtain total dry weight (g), which
111 included a previously measured cup weight. Cup weight was subtracted from this value to obtain
112 the dry weight of each individual.

113 2.3 Statistical analyses

114 The Ingestion Rate (IR) of each individual was measured using both pre- and post-
115 experiment algal cell counts, adjusted for controls, as:

$$116 \quad IR = \frac{[(E_0 - E_t) - (C_0 - C_t)]}{t}$$

117 where E_0 and E_t represent experimental algal cell concentrations at times 0 and t , respectively, C_0
 118 and C_t represent control algal cell concentrations at times 0 and t , and t is experimental duration
 119 (1 hour). $C_0 - C_t$ was included to adjust temporal changes in algal concentration due to algal
 120 growth or sedimentation in controls. Pre- and post-experiment algal concentrations were also
 121 used to determine the Clearance Rate (CR) of each individual as:

$$122 \quad CR = V \frac{[\ln(E_0 / E_t) - \ln(C_0 / C_t)]}{t}$$

123 where V represents the volume of suspension. As with IR, a correction factor was included in
 124 CR to account for changes in control tanks where no animals were present (Coughlan 1969).

125 Four of six calculated IR and CR values for *C. intestinalis* at the highest algal
 126 concentration (133084 cells/mL) were negative, possibly owing to a combination of sinking algal
 127 cells and/or less active animal feeding. Consequently, we removed this algal density from
 128 analysis for both species. Seven other negative values for IR and CR were also found for *C.*
 129 *intestinalis* and subsequently discarded when performing analysis. The reason for this problem
 130 is not clear, as we attempted to minimize disturbance and stress on animals prior to all feeding
 131 trails. We conducted two three-way ANOVA tests with the factors species, animal mass, food
 132 density, and their interactions to determine which factors affected IR and CR. Statistical analyses
 133 were performed in R-3.5.0 (R Core Team 2018) and RStudio 1.1.447 (RStudio Team 2016).

134 Animal functional response to varying food concentration was modeled using the FRAIR
 135 package in R-3.5.0 (Pritchard 2017). As food was not replaced after consumption, Rogers'
 136 random predator equation was used to describe the type II functional response of *C. intestinalis*
 137 (Barrios-O'Neill et al. 2014, Rogers 1972):

138
$$N_e = N_0(1 - \exp(a(N_e h - T)))$$

139 where N_e is the number of food items consumed, N_0 is the initial concentration of algal cells, a is
140 attack rate, h is handling time, and T is experimental duration. As the ingestion rate of *C. robusta*
141 generally increased linearly with increasing food concentration, a type I fit was applied to the
142 consumption data (FRAIR; Pritchard 2017):

143
$$N_e = N_0(aT)$$

144 These models were then non-parametrically bootstrapped (n = 2000) to generate 95% confidence
145 intervals for the functional response curves.

146 Relative Impact Potential (RIP; Dick et al. 2017) was calculated for co-occurring
147 ascidians using relative field abundance data from Brittany, France. For each instance in which
148 the species co-occurred (130 in total; Bouchemousse et al., unpublished data, see Bouchemousse
149 et al. (2017) for the sampling protocol) we obtained the ratio of relative abundance (A; measured
150 as Ind./m²) of *C. robusta* to *C. intestinalis*. We then randomly drew (from between three and six
151 measures per species) an IR for the invader and another for the native species. Relative Impact
152 Potential (RIP) was then estimated as the product of a randomly drawn A (from 130 co-
153 occurrence cases) and a randomly drawn IR ratio (invader IR divided by native IR) for that food
154 concentration. Results were bootstrapped 10000 times incorporating different combinations of A
155 and IR. Similar calculations were repeated for each food concentration. In Dick et al.'s (2017)
156 original formulation of RIP, abundance was measured directly and utilized maximum feeding
157 rate (1/h) from functional responses. Any combination of A and IR that yields a RIP>1 indicates
158 a greater relative impact by the non-native species, while those <1 indicate greater impact by the
159 native species. In this paper, we utilize individual IRs at different food concentrations to
160 estimate feeding rather than maximum feeding rate.

161

162 **3. Results**

163 *C. robusta* individuals tended to be slightly larger than co-occurring *C. intestinalis* and
164 also exhibited greater dispersion in dry mass (Fig. 1). However, mass was not a significant factor
165 contributing to differences in ingestion rates ($F_{1,51} = 0.07$, $P = 0.7920$), although its effect on
166 clearance rate was considerable ($F_{1,51} = 8.76$, $P = 0.0047$).

167 *C. robusta* had higher ingestion rates ($F_{1,51} = 6.9$, $P = 0.011$) and clearance rates ($F_{1,51} =$
168 19.2 , $P < 0.001$) than *C. intestinalis* (Fig. 2). Food concentration was a strong predictor of
169 ingestion rates ($F_{1,51} = 73.4$, $P < 0.001$), though it had no effect on clearance rate ($F_{1,51} = 0.9$, $P =$
170 0.353). Ingestion rate was also affected by a species \times food concentration interaction, with *C.*
171 *robusta* increasing at a faster rate with increasing food level (Figs. 2, 3). A similar pattern was
172 not apparent with species clearance rates ($F_{1,51} = 2.1$, $P = 0.154$). Ingestion rate ($F_{1,51} = 6.7$, $P =$
173 0.013) and clearance rate ($F_{1,51} = 4.5$, $P = 0.038$) also exhibited a significant mass \times food
174 concentration interaction.

175 Functional responses of the two ascidian species differed substantially. The invader, *C.*
176 *robusta*, exhibited a more profound increase in ingestion rate with increasing food level,
177 consistent with a type I functional response (Fig. 3). The native species, *C. intestinalis*,
178 displayed an asymptotic functional response to increasing food level, conforming to a type II
179 curve. Modelling with the FRAIR package in R-3.5.0 allowed for the determination of feeding
180 parameters for both species. Ingestion by *C. intestinalis* was best described using the Rogers'
181 random predator equation for type II functional responses, resulting in an attack rate $a = 0.124$ (P
182 < 0.001) and handling time $h = 8.294 \times 10^{-5}$ ($P < 0.001$). In contrast, *C. robusta* had a

183 substantially higher attack rate ($a = 0.196$, $P < 0.001$) and a negligible handling time,
184 characteristic of type I functional responses.

185 Field abundance data in Brittany, France, where the species co-occur illustrate that *C.*
186 *intestinalis* occurs more commonly than *C. robusta*. In over 361 quadrats studied, there were 188
187 instances where only the former species was present, three cases where only *C. robusta* occurred,
188 130 cases where species co-occurred, and 40 cases where neither species was found. When the
189 species co-occurred, bootstrapped seasonal abundance data revealed dominance by the
190 introduced species (mean ratio A of 1.31 of *C. robusta* to *C. intestinalis*). IR ratios ranged
191 between 1.27 and 7.92 (mean 4.46), indicating higher feeding rates by *C. robusta*. RIP values
192 (mean 5.77) indicate that *C. robusta* typically had a much greater relative impact. RIP values
193 were generally higher at higher food concentrations, with mean bootstrapped values of 1.89, 7.10,
194 1.61, 10.13, 6.62 and 7.25 across food concentrations of 1508, 3380, 5900, 12873, 29539 and
195 51616 cells/mL, respectively. Most of *C. robusta*'s greater feeding impact was attributable to its
196 higher IR, though higher abundance was also important.

197

198 4. Discussion

199 Ascidians have experienced many biological invasions and concomitant range expansions
200 in recent decades (e.g. Gittenberger 2007; Lambert 2007, 2009; Ruis et al. 2012; Ordóñez et al.
201 2013; Zhan et al. 2015; Bullard and Carmen 2016; Simkanin et al. 2016; Nydam et al. 2017).
202 The English Channel is no exception, with numerous reports of introduced ascidians including
203 *Botrylloides violaceus*, *B. diegensis*, *Aplidium glabrum*, *Diplosoma listerianum*, *Molgula*
204 *complanata*, *Corella eumyota*, *Perophora japonica*, *Styela clava*, *Didemnum vexillum*,
205 *Asterocarpa humilis* and *Ciona robusta* established on both sides of the channel (Gittenberger

206 2007; Zhan et al. 2010; Minchin et al. 2013; Bishop et al. 2015). *C. robusta* was long confused
207 with *C. intestinalis*, though they are genetically and morphologically distinct and seemingly
208 reproductively isolated (Zhan et al. 2010; Sato et al. 2012; Brunetti et al. 2015; Bouchemousse et
209 al. 2016a). As the species co-occur on both sides of the English Channel it is possible that they
210 compete for settlement substrates, though Bouchemousse et al. (2017) reported highly variable
211 settlement and suggested environmental variation modulated competition. It is also possible the
212 species compete for food at times. Our study revealed significant feeding rate differences
213 between the species, with introduced *C. robusta* exhibiting higher ingestion rates and clearance
214 rates than native *C. intestinalis* (Fig. 2a,b). Differences in ingestion rate were more pronounced
215 as food concentration increased, suggesting high food levels in nature could favor the introduced
216 species.

217 Functional responses of *C. robusta* and *C. intestinalis* also differed, conforming to type I
218 and II curves, respectively (Fig. 3). Type II curves and higher FR of the introduced species are
219 consistent with an array of aquatic and terrestrial invertebrates, fishes and with plants (Dick et al.
220 2017). The greater FR of *C. robusta* was largely responsible for its higher RIP scores. Thus,
221 when the species co-occur, *C. robusta* should have a greater impact on algal foods than *C.*
222 *intestinalis*. However, co-occurrence of the species in nature is less common (130 occurrences)
223 than instances where *C. intestinalis* (188 cases) occurs alone, thus the regional impact of the
224 invader will be muted by its more confined distribution. We expect that impact exerted by *C.*
225 *robusta* would expand commensurate with the extension of its distribution.

226 Clearance rates have been well-studied in ascidians (see Jacobi et al. (2018) and
227 references cited therein). The absence of an apparent asymptote in feeding rate in *C. robusta*
228 (Fig. 3 dashed line) was surprising given the broad range of food concentrations provided to

229 study animals. However, Pascoe et al. (2007) observed that *C. intestinalis* exhibited a strong
230 positive relationship between ingestion rate and food concentration up to 10^6 cells/mL of
231 *Isochrysis galbana*. Armsworthy et al. (2001) also noted that IR increased linearly with food
232 concentration in the ascidian *Halocynthia pyriformis*. Pascoe et al. (2007) also revealed that CR
233 was maximal around 5×10^3 cells/mL, whereas we did not observe a clear maximum for either
234 species tested (Fig. 2b). Typically with filter-feeders, a maximum algal concentration is reached
235 (i.e. Incipient Limiting Level) beyond which IR is constant and CR declines exponentially (e.g.
236 Robbins 1983; Petersen and Riisgård 1992; Sigsgaard et al. 2003; Petersen 2007). At high
237 suspended particulate load, a reduction in IR associated with satiation (Pascoe et al. 2007) could
238 be active rejection (ie. squirting; Robbins 1984; Petersen 2007) or a reduction in lateral cilia beat
239 frequency (Petersen 2007). It is important to note that we also did not observe a decline in CR at
240 very low food levels, which some authors attributed to reduced cilia beating in the branchial
241 basket (Petersen et al. 1999).

242 Our study had some unexpected IR, and hence CR, results. For example, from an initial
243 39 total observations, a majority of IR and CR values for *C. intestinalis* at the highest food
244 concentration were negative and thus removed. We likewise removed high food concentration
245 feeding results for *C. robusta* even though this species was not plagued by this issue. A number
246 of other studies have observed a reduction in clearance rate with increasing food concentration
247 (Petersen and Riisgård 1992; Petersen et al. 1999). At high food concentrations, the gut reaches
248 an intake threshold above which the clearance rate is reduced as a form of protection against gut
249 saturation (see Petersen and Riisgård 1992; Petersen et al. 1999; Petersen 2007). Petersen (2007)
250 recommended an acclimation period of 20-140 min prior to measuring clearance rates. While our
251 experiment did not incorporate a formal adjustment period, we commenced experiments only

252 after animals appeared robust with seemingly normal feeding behaviour. Seven of the 39 trials
253 conducted also yielded negative IR and CR results, though the problem was limited to *C.*
254 *intestinalis* predominantly at the three lowest food concentrations. These cases were removed
255 prior to statistical analysis, though the reason for the aberrant results remains unclear.

256 Clearly, comparative *per capita* studies of invasive versus native species, plus proxies for
257 numerical responses such as abundance, can rapidly inform actual or potential ecological impacts
258 of invasions (Dick et al. 2017a,b). Here, with our ascidian example, these methods are congruous
259 with field patterns of invasions and ecological impact, including competition and species
260 displacement. We encourage further development of these metrics across taxonomic and trophic
261 groups, and incorporation of context-dependencies, such as temperature change with climate
262 change (Dick et al. 2017b). This will allow invasion ecology to become truly predictive, with
263 opportunities to focus limited resources on the most harmful actual and potential invaders.

264

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273

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393 **Figure Legends**

394 Figure 1. Ingestion rate (a) and clearance rate (b) of native (filled circles) and invasive (open
395 circles) *Ciona* individuals as a function of animal dry mass. Note the log scale for both
396 graphs.

397 Figure 2. Ingestion rate (a) and clearance rate (b) of *Ciona* individuals as a function of *Isochrysis*
398 *affinis galbana* concentration. Overlapping points in b have been slightly offset to the
399 right for clarity. Note the log scale for ingestion rate (a).

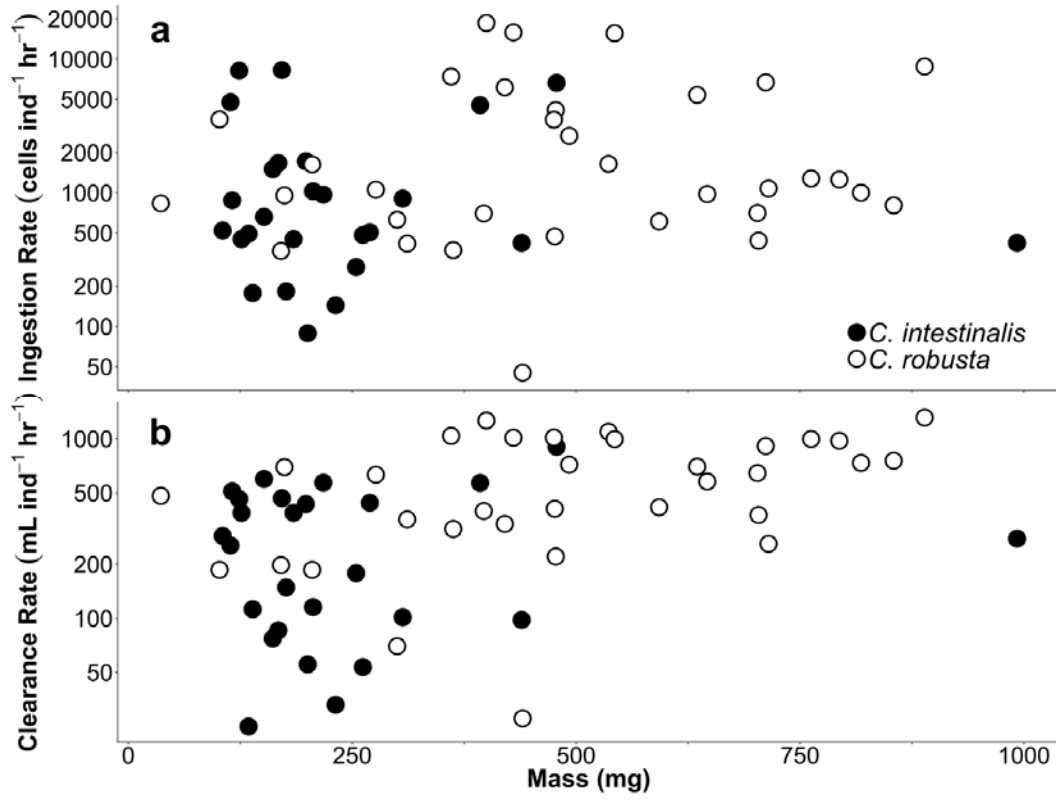
400 Figure 3. Fitted functional responses of native (solid line) and introduced (dashed) *Ciona* (with
401 95% CI bands). *C. robusta* statistically conforms to a type I functional response, while *C.*
402 *intestinalis* conforms to a type II response.

403

404 Figure 1

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406

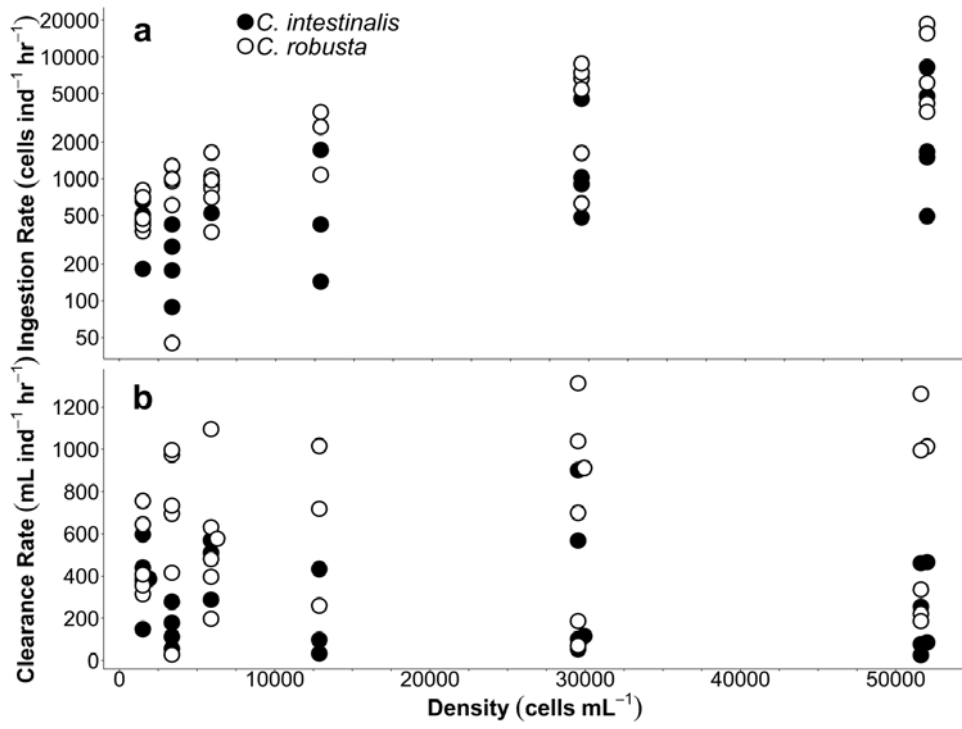


407

408 Figure 2

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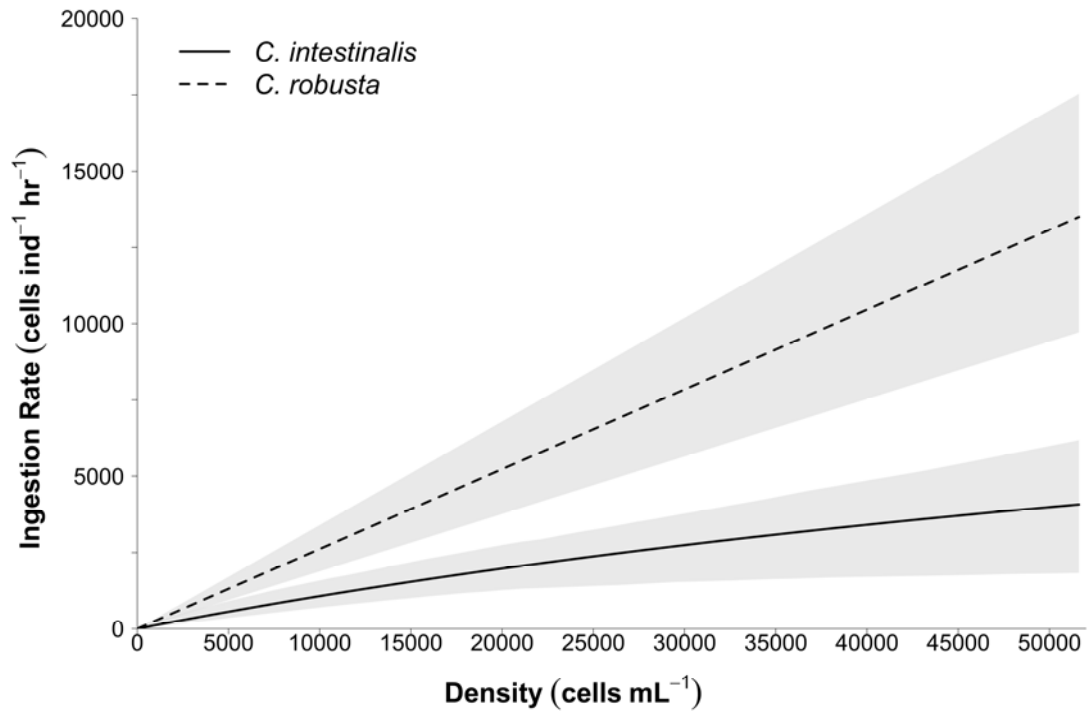
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411

412 Figure 3

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