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## Comparative feeding rates of native and invasive ascidians

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## ABSTRACT

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

## 1. Introduction

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

Ascidians are primarily sessile organisms with a brief pelagic (le-cithotrophic) larval stage. Numerous ascidian species have experienced an increase in global range linked to human-mediated spread and, in some cases, climate warming (e.g. Lambert, 2001, 2007; Sorte et al., 2010; Bock et al., 2011; Zhan et al., 2015; Simkanin et al., 2016; Nydam et al., 2017). In total, Zhan et al. (2015) catalogued 80 species that were recognized as non-native in the habitats in which they were reported. In the Netherlands, non-native ascidians colonized during two major spates, one in 1974–1977, the other in 1991–2004 (Gittenberger, 2007). The increase in the number of non-native ascidian species

reported in the USA was low but relatively linear between 1850 and 1950, thereafter increasing sharply on both Pacific and Atlantic coasts (Simkanin et al., 2016).

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

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

Identifying which introduced species are likely to produce strong impacts is a daunting challenge owing to the varying nature of the

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species themselves, the nature of the ecosystems that they are introduced into, and a variety of context-dependencies (Kumschick et al., 2015). Recently, Dick et al. (2014) proposed analyzing comparative functional responses (FR) of introduced species (or those that might be introduced) versus those of comparable native taxa to assess whether the former would have high impact. The functional response considers a species' per capita resource consumption as a function of resource availability, and Dick et al.'s (2014) study highlighted that invader FRs typically exceed those of native species. This approach was then extended by combining it with species abundance data to yield a total impact potential for the invader scaled to that of the native species (Dick et al., 2017a).

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

## 2. Methodology

### 2.1. Sampling procedure

*Ciona* individuals were collected by scraping from pontoons and pillars in Brest, France on 25th September 2015 and acclimated in the animal husbandry lab (18 °C) at the Roscoff Biological Station, France. *Ciona* cultures were maintained on *Isochrysis affinis galbana* algae (issued from the Roscoff Culture Collection (RCC) facility under the reference numbers RCC1349) at concentrations of 15–20 × 10<sup>6</sup> cells/mL. Seven experimental food concentration treatments (1508, 3380, 5900, 12,873, 29,539, 51,616, 133,084 cells/mL) were determined using a Malassez cell counting chamber. Experimental *Ciona* individuals were housed in separate cylindrical tanks, each filled with 2 L seawater, totaling seven tanks. Six of the seven tanks contained *Ciona* individuals, with the remaining one containing only *Isochrysis affinis galbana*, thereby serving as a control. Of the six experimental tanks, three contained a single *C. intestinalis* individual and food, while the other three had a single *C. robusta* individual and food. *Ciona* individuals were randomly selected and distinguished morphologically. This design was repeated twice to produce six replicates for each ascidian species at each algal concentration, with the exception of the treatment with 12,783 algal cells/mL, where time constraints only allowed for three replicates of each species.

### 2.2. Experimental setup

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

### 2.3. Statistical analyses

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

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

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

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

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

Four of six calculated IR and CR values for *C. intestinalis* at the highest algal concentration (133,084 cells/mL) were negative, possibly owing to a combination of sinking algal cells and/or less active animal feeding. Consequently, we removed this algal density from analysis for both species. Seven other negative values for IR and CR were also found for *C. intestinalis* and subsequently discarded when performing analysis. The reason for this problem is not clear, as we attempted to minimize disturbance and stress on animals prior to all feeding trials. We conducted two three-way ANOVA tests with the factors species, animal mass, food density, and their interactions to determine which factors affected IR and CR. Statistical analyses were performed in R-3.5.0 (R Core Team, 2018) and RStudio 1.1.447 (RStudio Team, 2016).

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

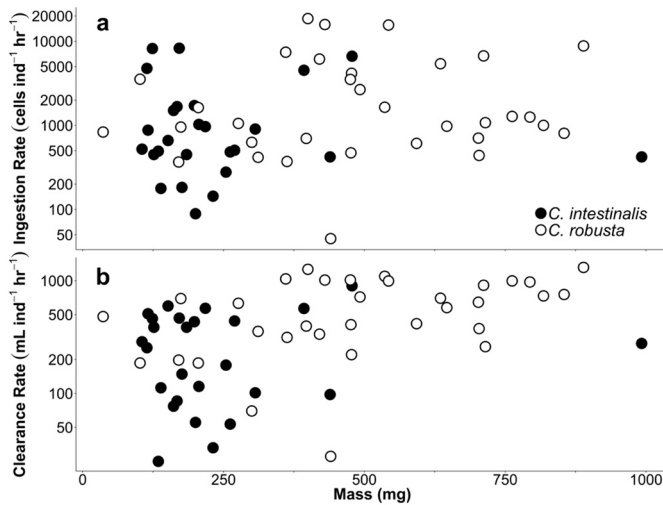
$$N_e = N_0(1 - \exp(-a(N_e h - T)))$$

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

$$N_e = N_0(aT)$$

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

Relative Impact Potential (RIP; Dick et al., 2017a) was calculated for co-occurring ascidians using relative field abundance data from Brittany, France. For each instance in which the species co-occurred (130 in total; Bouchemousse et al., unpublished data, see Bouchemousse et al. (2017) for the sampling protocol) we obtained the ratio of relative abundance ( $A$ ; measured as Ind./m<sup>2</sup>) of *C. robusta* to *C. intestinalis*. We then randomly drew (from between three and six measures per species) an IR for the invader and another for the native species. Relative Impact Potential (RIP) was then estimated as the product of a randomly drawn  $A$  (from 130 co-occurrence cases) and a randomly drawn IR ratio (invader IR divided by native IR) for that food concentration. Results were bootstrapped 10,000 times incorporating different combinations of  $A$  and IR. Similar calculations were repeated for each food concentration. In Dick et al. (2017a)'s original formulation of RIP, abundance was measured directly and utilized maximum feeding rate (1/h) from functional responses. Any combination of  $A$  and IR that yields a RIP > 1 indicates a greater relative impact by the non-



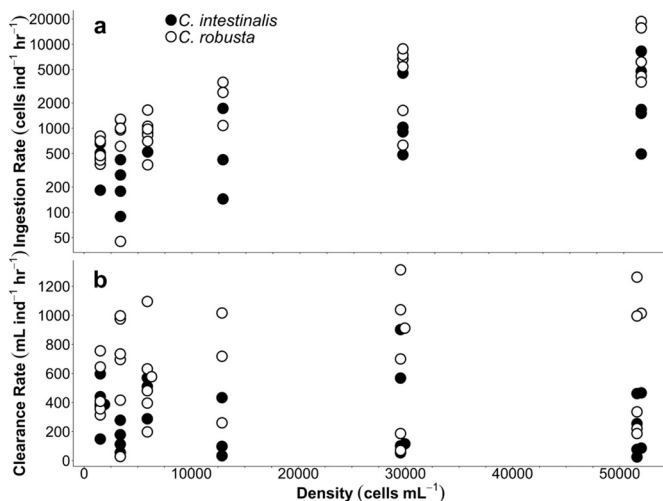
**Fig. 1.** Ingestion rate (a) and clearance rate (b) of native (filled circles) and invasive (open circles) *Ciona* individuals as a function of animal dry mass. Note the log scale for both graphs.

native species, while those < 1 indicate greater impact by the native species. In this paper, we utilize individual IRs at different food concentrations to estimate feeding rather than maximum feeding rate.

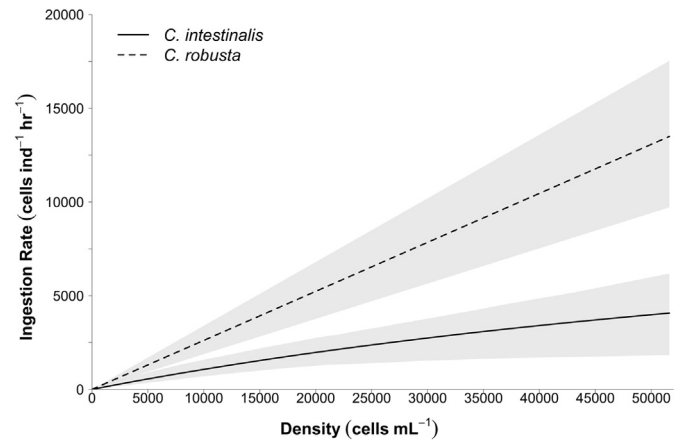
### 3. Results

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

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



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



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

$P = 0.038$ ) also exhibited a significant mass  $\times$  food concentration interaction.

Functional responses of the two ascidian species differed substantially. The invader, *C. robusta*, exhibited a more profound increase in ingestion rate with increasing food level, consistent with a type I functional response (Fig. 3). The native species, *C. intestinalis*, displayed an asymptotic functional response to increasing food level, conforming to a type II curve. Modeling with the FRAIR package in R-3.5.0 allowed for the determination of feeding parameters for both species. Ingestion by *C. intestinalis* was best described using the Rogers' random predator equation for type II functional responses, resulting in an attack rate  $a = 0.124$  ( $P < 0.001$ ) and handling time  $h = 8.294 \times 10^{-5}$  ( $P < 0.001$ ). In contrast, *C. robusta* had a substantially higher attack rate ( $a = 0.196$ ,  $P < 0.001$ ) and a negligible handling time, characteristic of type I functional responses.

Field abundance data in Brittany, France, where the species co-occur illustrate that *C. intestinalis* occurs more commonly than *C. robusta*. In 361 quadrats studied, there were 188 instances where only the former species was present, three cases where only *C. robusta* occurred, 130 cases where species co-occurred, and 40 cases where neither species was found. When the species co-occurred, bootstrapped seasonal abundance data revealed dominance by the introduced species (mean ratio A of 1.31 of *C. robusta* to *C. intestinalis*). IR ratios ranged between 1.27 and 7.92 (mean 4.46), indicating higher feeding rates by *C. robusta*. RIP values (mean 5.77) indicate that *C. robusta* typically had a much greater relative impact. RIP values were generally higher at higher food concentrations, with mean bootstrapped values of 1.89, 7.10, 1.61, 10.13, 6.62 and 7.25 across food concentrations of 1508, 3380, 5900, 12,873, 29,539 and 51,616 cells/mL, respectively. Most of *C. robusta*'s greater feeding impact was attributable to its higher IR, though higher abundance was also important.

### 4. Discussion

Ascidiaceans have experienced many biological invasions and concomitant range expansions in recent decades (e.g. Gittenberger, 2007; Lambert, 2007, 2009; Ruis et al., 2012; Ordóñez et al., 2013; Zhan et al., 2015; Bullard and Carman, 2016; Simkanin et al., 2016; Nydam et al., 2017). The English Channel is no exception, with numerous reports of introduced ascidians including *Botrylloides violaceus*, *B. diegensis*, *Aplidium glabrum*, *Diplosoma listerianum*, *Molgula complanata*, *Corella eumyota*, *Perophora japonica*, *Styela clava*, *Didemnum vexillum*, *Asterocarpa humilis* and *Ciona robusta* established on both sides of the channel (Gittenberger, 2007; Zhan et al., 2010; Minchin et al., 2013; Bishop et al., 2015). *C. robusta* was long confused with *C. intestinalis*,

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

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

Clearance rates have been well-studied in ascidians (see Jacobi et al. (2018) and references cited therein). The absence of an apparent asymptote in feeding rate in *C. robusta* (Fig. 3 dashed line) was surprising given the broad range of food concentrations provided to study animals. However, Pascoe et al. (2007) observed that *C. intestinalis* exhibited a strong positive relationship between ingestion rate and food concentration up to  $10^6$  cells/mL of *Isochrysis galbana*. Armsworthy et al. (2001) also noted that IR increased linearly with food concentration in the ascidian *Halocynthia pyriformis*. Pascoe et al. (2007) also revealed that CR was maximal around  $5 \times 10^3$  cells/mL, whereas we did not observe a clear maximum for either species tested (Fig. 2b). Typically with filter-feeders, a maximum algal concentration is reached (i.e. Incipient Limiting Level) beyond which IR is constant and CR declines exponentially (e.g. Robbins, 1983; Petersen and Riisgård, 1992; Sigsgaard et al., 2003; Petersen, 2007). At high suspended particulate load, a reduction in IR associated with satiation (Pascoe et al., 2007) could be active rejection (ie. squirting; Robbins, 1983; Petersen, 2007) or a reduction in lateral cilia beat frequency (Petersen, 2007). It is important to note that we also did not observe a decline in CR at very low food levels, which some authors attributed to reduced cilia beating in the branchial basket (Petersen et al., 1999).

Our study had some unexpected IR, and hence CR, results. For example, from an initial 39 total observations, a majority of IR and CR values for *C. intestinalis* at the highest food concentration were negative and thus removed. We likewise removed high food concentration feeding results for *C. robusta* even though this species was not plagued by this issue. A number of other studies have observed a reduction in clearance rate with increasing food concentration (Petersen and Riisgård, 1992; Petersen et al., 1999). At high food concentrations, the gut reaches an intake threshold above which the clearance rate is reduced as a form of protection against gut saturation (see Petersen and Riisgård, 1992; Petersen et al., 1999; Petersen, 2007). Petersen (2007) recommended an acclimation period of 20–140 min prior to measuring clearance rates. While our experiment did not incorporate a formal adjustment period, we commenced experiments only after animals appeared robust with seemingly normal feeding behaviour. Seven of the 39 trials conducted also yielded negative IR and CR results, though the problem was limited to *C. intestinalis* predominantly at the three lowest food concentrations. These cases were removed prior to statistical analysis, though the reason for the aberrant results remains unclear.

Clearly, comparative per capita studies of invasive versus native

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

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