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Protozoan functional responses: effects of species, genotype, and anti-predator defences

Shelly Duquette, Res Altwegg‡ and Bradley R. Anholt*

Department of Biology, University of Victoria, PO Box 3020 Stn CSC, Victoria, BC V8W 3N5, Canada

ABSTRACT

Hypothesis: Induced anti-predator defences reduce foraging efficiency.

Organism: Two clones of *Euplotes aediculatus*, and one clone of *E. plumipes*, species of hypotrich ciliated protozoan feeding on the green alga *Chlorella vulgaris*.

Study site: Laboratory experiment.

Methods: We induced defences using frozen turbellarian flatworm predators. We counted captured cells directly using the autofluorescence of chlorophyll. We then estimated functional responses of induced and uninduced morphs using non-linear mixed effects models of counts of captured cells.

Results: All clones had Type II functional responses. Variation in attack rates and handling times was as large between clones as between species. Only the clone with the largest morphological change clearly had reduced foraging efficiency. This was due to longer handling times.

Keywords: *Chorella*, chlorophyll autofluorescence, *Euplotes*, model selection, phenotypic plasticity, *Stenostomum*.

INTRODUCTION

Prey defences that are inducible by predator cues are widespread in natural systems (Lima, 1998). They have been shown repeatedly to decrease predation mortality and therefore affect vital rates of predators and prey. To accurately describe the dynamic behaviour of natural food webs, there is a clear need to incorporate inducible defences into theoretical predator–prey models and to test experimentally their predictions. Although there are many well-characterized natural systems with inducible defences, less research has been directed at their influence on dynamic relationships.

Ecological studies have revealed the ubiquity of inducible defences and their consequences for communities (Werner and Peacor, 2003), but much of the existing theory describes how they affect the predator's ability to control prey populations or how they change competitive

* Author to whom all correspondence should be addressed. e-mail: banholt@uvic.ca

‡ *Present address*: Avian Demography Unit, Department of Statistical Sciences, University of Cape Town, Rondebosch 7701, Republic of South Africa.

interactions among prey. Less is known about the propagation of trait-mediated indirect effects of induced defences through food webs (Werner and Anholt, 1996; Schmitz, 2000), and almost nothing is known about how they influence community stability. Measurement of functional response parameters can inform our understanding of the nature and magnitude of consumer–resource interactions and the effect of inducible defences on these relationships. These data are essential for modelling population dynamic consequences of induced responses. Existing theory argues that inducible defences are inherently stabilizing (Anholt and Werner, 1999). When defences are more strongly expressed at high densities of predators, predation rates will decline with increasing predator density. When defences are less strongly expressed at high prey densities because of reduced resources, as they are in *Euplotes* (e.g. Wiackowski and Szkarlat, 1996), predation rates will increase with prey density. These density-dependent feedbacks should be stabilizing, provided that they do not have long time-lags (Luttbeg and Schmitz, 2000).

Measurement of the effect of inducible defences on the interaction between species in the food web is fundamental for the analysis of stability in a wide range of ecosystems. With few exceptions (e.g. Abrams and Vos, 2003; Vos *et al.*, 2004a, 2004b), predator–prey models ignore the complications associated with inducible defences, and models that include them focus on the defended prey and its predator and exclude prey resources. There is evidence, however, that expression of defence can alter the relationship between the inducible prey and its own resources (Ramcharan *et al.*, 1992; Trussell *et al.*, 2003). This results in indirect interactions with other members of the food web (e.g. Werner and Anholt, 1996).

Typically, defended prey have lower rates of somatic or population growth (Werner and Peacor, 2003). Such costs are predicted by theory (Harvell, 1990), which argues that in the absence of costs, evolution selects for constitutive defences. A reduced rate at which defended prey can consume their own food, caused by changes in morphology or behaviour, is one such cost. The relationship between resource intake and resource density is described by the functional response. Differences in functional response parameters between defended and undefended consumers therefore reflect ecological costs of defence and allow the integration of these costs into consumer–resource models. The shape of the functional response is determined by characteristics of the consumer–resource relationship and affects its inherent stability. The distinction between the asymptotic Type II and the sigmoid Type III responses (Holling, 1959) is particularly significant. In Type III responses, the range over which the proportion of resources consumed initially increases with resource density inherently stabilizes the system. Type II responses are destabilizing because the proportion of resources consumed declines with density for all resource densities.

In this study, we examine the relationship between consumption rate and resource density in a protozoan model system where consumers express inducible defences. The ciliate *Euplotes* is ordinarily a flattened ovoid cell but, within a few hours of exposure to predators like the turbellarian flatworm *Stenostomum*, it modifies its shape by increasing the cell width (Kuhlmann and Heckmann, 1985; Duquette *et al.*, 2005). This process requires considerable energy and protein for cytoskeletal microtubules (Jerka-Dziadosz *et al.*, 1987) and costs *Euplotes* a delay in cell division. Cell division resumes following morphological transformation, but population growth rates are lower in defended cells (Kusch and Kuhlmann, 1994). Little is known about how this cost is mediated, but altered foraging is expected to play a role because of the close relationship between population growth and food intake. *Euplotes* ciliates are motile filter feeders. Cells extract particles from the aqueous medium using the adoral zone of membranelles, a highly specialized oral ciliature that creates fluid currents to direct food

(mainly smaller protists and bacteria) towards the cytostome. Ingestion is via phagocytosis, with volume limiting food vacuole packing (Dolan and Coats, 1991). The membrane supply process for food vacuole formation is generally expected to limit the rate of food intake (Fenchel, 1986) and accounts for saturation in the functional response of *Euplotes*. Modelling the shape of the functional response requires data for individual food intake, measured over a range of prey concentrations that includes saturating densities.

Feeding rates for microzooplankton have been estimated using the change in food concentration over hours or days of incubation with consumers (Landry and Hassett, 1982). These assays require relatively high food resource densities, which change appreciably during the experiment. A change in food density complicates estimation of the functional response because the main explanatory variable is not constant within experimental units. One solution is direct counting of fluorescently labelled food items (Sherr *et al.*, 1987) over short incubation times (minutes) so that prey depletion can be ignored. Prey can be live-stained, but it can change cell surface properties in ways that decrease palatability to predators (Sanders, 1988). Alternatively, unstained algae can also be detected after ingestion taking advantage of epifluorescence due to the autofluorescence of chlorophyll (e.g. Premke and Arndt, 2000).

Here, we directly counted ingested algae within defended and undefended cells of three *Euplotes* clones from two species that had been allowed to forage at a range of algae concentrations. We then considered the three basic functional responses originally proposed by Holling (1959), to determine which one best described the effect of prey density on the predation rate. We also tested whether the functional response was modified by the inducible defence.

MATERIALS AND METHODS

Model organisms and culture procedures

We compared the functional response of clones within two species of *Euplotes* to their resources in the presence and absence of cues from the turbellarian predator *Stenostomum virginianum* Nuttycombe 1931. *Euplotes aediculatus* Pierson 1943 (Clones 1 and 2) and *E. plumipes* Stokes 1884 (Clone 3) were obtained with thanks from K. Wiackowski (Jagiellonian University, Krakow, Poland). We used the unicellular green algae *Chlorella vulgaris* (University of Toronto Culture Collection, Strain 266) as resources.

All *Euplotes* isolates were maintained as clonal populations in a liquid medium consisting of 0.04% crushed protozoan pellets (Nr. 13-2360, Carolina Biological Supply Company, NC, USA) in NAYA spring water filtered through double-layered No. 4 coffee filters (Thrifty Foods Inc.). Two wheat grains in 300 ml of medium were sterilized by autoclaving, then inoculated with 50–100 µl of a mid-log phase liquid culture of *Bacillus cereus* (Boreal Laboratories, St. Catherines, ON, Canada) in tryptic soy broth (Difco Laboratories, Detroit, MI, USA), to provide a food source for the ciliates. *Chlorella vulgaris* was cultured at 24°C in Bold's Basal Medium under constant illumination (Philips F20T12/ww 20 W full spectrum fluorescent tubes). Filtered air (0.22 µm) was bubbled through the algal cultures to keep the algae and nutrients well distributed.

We isolated the predatory flatworm *Stenostomum virginianum* (Rhabdocoela: Turbellaria) from sediments of a freshwater pond on the University of Victoria campus. Asexually reproducing populations were established and raised in batch culture on spring water (NAYA, Mirabel, PQ, Canada) and sterilized wheat grains in 100×50 mm Pyrex

crystallizing dishes. Cultures contained significant numbers of bacteria and a small flagellate, both of which were isolated along with the worms from the original pond and provided a food source for them. To eliminate the effects of predation on *Euplotes* in this experiment, we used freezer-killed *Stenostomum* to induce morphological changes in *Euplotes* (Altwegg *et al.*, 2004).

Functional response of *Euplotes*

We established completely randomized blocks of experimental replicates in 24-well tissue culture plates (Costar, Corning). There were nine temporal blocks, each set up on different dates. *Euplotes* cells were re-suspended from well-established cultures into fresh sterile medium to wash away bacteria and 60 individuals were counted into experimental wells in 200 µl of fluid. We then added either 200 µl of freezer-killed worms (200 worms $\cdot$ ml^{-1}, –4°C) (defended treatments) or 200 µl of NAYA water (control/undefended treatments). The experimental plates were then incubated at room temperature for 24 h to allow induction of defensive morphology to take place in the defended treatment wells.

We took photomicrographs of randomly sampled live *Euplotes* cells from five replicate wells (data were missing for Clone 3 in one of these replicates) with an inverted microscope (Leica DM-IRB) and an attached CCD camera (COHU) using Image Pro Plus 4.5 software. We measured the maximum cell width from the photographs to compare the level of defence among the three clones.

To assay the predation rate, we added 200 µl of *Chlorella vulgaris* culture to each experimental well. The final concentrations of algal cells ranged from 1×10^7 cells $\cdot$ ml^{-1} to 3.13×10^5 cells $\cdot$ ml^{-1} over a 1/2 dilution series, for a total of six prey levels. This concentrated the experimental effort at low prey densities, as this is the region of the functional response where the distinction between Type II and Type III responses occurs. While this experimental design results in a greater chance of detecting differences in the attack rate, it has somewhat reduced power to detect differences in the asymptote, which is reached only at higher prey densities.

Euplotes were allowed to feed on the algae for 16 min, and the experiment was stopped by adding 6 µl of 20× diluted alkaline Lugol's solution. This was followed by 12 µl of borate-buffered formalin to preserve the cells and 7.2 µl of 4× diluted 3% sodium thiosulphate to clear any iodine staining from the Lugol's solution, which masks chlorophyll fluorescence (Sherr *et al.*, 1987). To prepare samples for epifluorescence microscopy, we transferred all of the preserved *Euplotes* cells in each well by pipette into 1 ml of sterile NAYA water while observing through a stereomicroscope (Leica M8). We washed the cells by successive transfers into 500 µl of clean NAYA water.

The first six cells encountered under the microscope were mounted on glass microscope slides in a drop of water under a cover slip. We examined the cells under epifluorescence (Zeiss Axioskop 2, Attoarc 2 100 W mercury lamp, FITC filter 450–490 nm) and took photographs with a digital camera (Q Imaging Microimager 2) using Northern Eclipse software. We were then able to count the number of algae that had been eaten in the 16 min of the experiment, as they were clearly visible within the cells (Fig. 1). The mean number of algal cells consumed by the six *Euplotes* was used as the estimate of consumption for each experimental unit to maintain the statistical independence of the estimates. Accidental loss of cells during preparation for epifluorescence microscopy resulted in some replicates being dropped from the analysis.

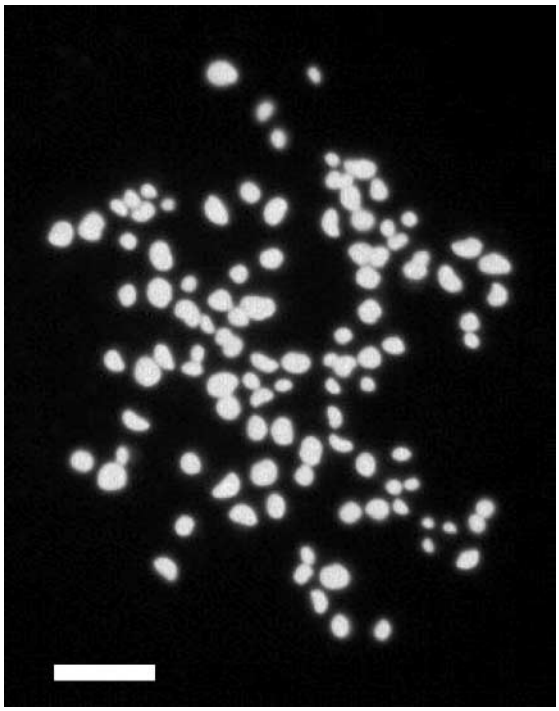

Fig. 1. Visualization of ingested algae inside *Euplotes* using epifluorescence microscopy. Image shown is *E. aediculatus* Clone 1. Cells were fixed with alkaline Lugol's iodine solution and borate-buffered formalin after 16 min of incubation with *Chlorella vulgaris* algae. Visualization at 400× (Zeiss epifluor 40× objective). Scale bar = 20 μm.

Statistical methods

The following equations were used to examine the effect of prey density (X) on the number of prey eaten (y):

$$\text{Type I, linear relationship:} \quad y(X) = a + bX \tag{1}$$

$$\text{Type II, asymptotic:} \quad y(X) = aX / (1 + abX) \tag{2}$$

$$\text{Type III, sigmoid:} \quad y(X) = aX^2 / (1 + abX^2) \tag{3}$$

An exponential version of the Type III functional response was initially considered but not examined further because the estimates would not converge when fitting the model.

Type I is a simple linear regression model. To test for the effect of defence, separate estimates of the slope and/or intercept were fit for the two treatments. We also fitted a Type I functional response with an asymptote using break point regression. This is a linear regression for values less than the breakpoint and the mean of the values above the breakpoint. The breakpoint is an additional parameter to be estimated. Type II is a hyperbolic function, where a is the slope of the curve at the origin (determined by the predator's attack rate) and $1/b$ is the asymptote (determined by b, the predator's handling time). Type III is a sigmoid modification of Model 2 where the attack rate increases with density.

We first determined which functional response best described the number of prey consumed and whether clones needed separately estimated parameters for an adequate description. We compared the fit of models to the data when all clones shared the same functional response, differed in only one parameter or differed in all parameters. After determining the best overall description, we tested within clones whether the functional response needed to be modified further to accommodate differences due to induction treatments.

We expected the parameter values to vary slightly among experimental blocks. Therefore, we used non-linear mixed effects models to fit the functional response equations. We associated random effects due to blocks for slope and asymptote terms in the models, but not the breakpoint of the breakpoint regression (see Altwegg *et al.*, 2006 for a more detailed description). All models were fit to the data by maximum likelihood using the nlme package in program R v2.20 (R Development Core Team, 2005). Comparison of the models was based on Akaike's information criterion (AIC), where lower AIC values indicate models that are better supported by the data (Burnham and Anderson, 2002). More complex models necessarily fit the data better than simpler ones, and AIC mediates a trade-off between the number of parameters and the residual variation. It has the added advantage that it permits comparison of models that are structurally dissimilar, such as among functional responses. Akaike weights were calculated and give the relative support for each model in the set. Ratios between Akaike weights of two models can be used as heuristic evidence ratios, to determine the relative strength of support for one model over another (Burnham and Anderson, 2002).

RESULTS

The three *Euplotes* clones initially had similar mean widths of about 73 μm. Exposure to predator cues increased cell width in all three clones, but not to the same extent. *Euplotes aediculatus* Clone 1 and *E. plumipes* Clone 3 had similar width maxima after induction but *E. aediculatus* Clone 2 was nearly 15 μm wider (Fig. 2).

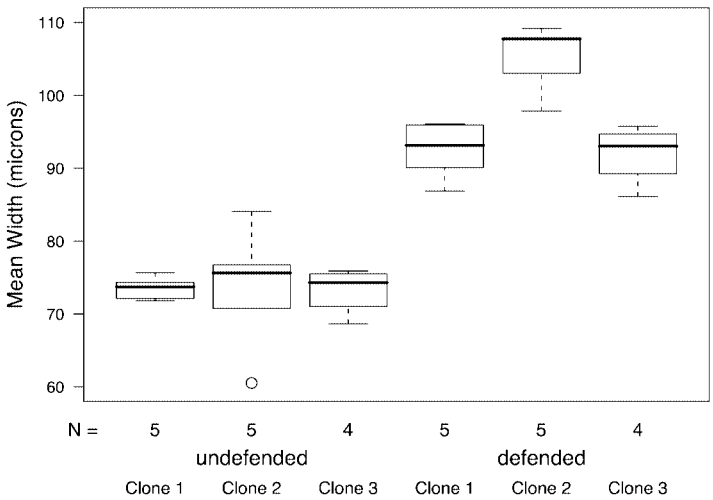

Fig. 2. Width of defended and undefended *Euplotes*. Defended *Euplotes* were measured 24 h after exposure to predator cues; independent data points are the means from within a single culture well.

Table 1. Model selection summary: Fit of functional response models for two clones of *Euplotes aediculatus* and one clone of *E. plumipes*

Model (shared parameters)	Log likelihood	*K*	AIC	ΔAIC	AIC weight
Type I (none)	−1313.62	9	2645.24	228.23	
Type I (slope)	−1365.49	3	2737.30	316.29	
Type I with breakpoint (none)	−1195.70	21	2435.41	18.40	<0.0001
Type I with breakpoint (slope)	−1241.89	9	2501.78	84.77	
Type I with breakpoint (asymptote)	−1247.44	9	2512.87	95.86	
Type I with breakpoint (all)	−1285.62	7	2585.25	168.24	
Type II (none)	**−1196.51**	**12**	**2417.01**	**0**	**>0.999**
Type II (attack rate)	−1227.86	8	2471.72	54.71	
Type II (handling time)	−1208.50	8	2433.29	16.28	<0.0001
Type II (all)	−1353.58	4	2721.16	304.15	
Type III (none)	−1223.41	12	2488.81	71.80	
Type III (all)	−1314.66	4	2643.31	226.30	

Note: All models were fitted using functions lme and nlme in program R under four conditions: (1) all clones shared the same functional response parameters, shared only (2) attack rate or (3) handling time, or (4) they shared no parameters. Lower AIC values (in **bold** type) indicate the best model. *K* is the number of parameters in the model.

When all three clones were analysed together and the effect of defence ignored, the best Type II model included differences in both the attack rate and the handling time among the three clones (Table 1). There was almost no support for models where clones differed in only one parameter. There was no support for the model where all clones shared an identical functional response (Fig. 3, dashed line).

In *E. aediculatus* Clone 2, induced cells had an increased handling time (9.6 s vs. 7.4 s) but there was no support for differences due to inducible defences in the functional response of *E. aediculatus* Clone 1, or in *E. plumipes* Clone 3 (Table 2). The deviance of Model 'Type II (all)' was close to that of 'Model II (none)', showing that the additional parameters for the effect of defence improved the model fit little. Note that a small difference in AIC does not necessarily indicate an equally good model in such cases, because adding one parameter with no explanatory power can increase AIC by a maximum of 2.

The attack rates for the three clones ranged from 1.95 in Clone 1 to 5.11 in Clone 3 (Table 3). The average maximum predation rate was 5.5 prey per minute per predator (Table 3, combined analysis), corresponding to an average handling time of 10.86 s per prey ingested.

Because the experimental design emphasized values at low densities to distinguish Type III functional responses, model fits are good at low densities but there are pronounced deviations at high densities.

DISCUSSION

This experiment has demonstrated clonal variation in the level of defence induction within species that can be larger than interspecific variation. In addition, the defended forms varied

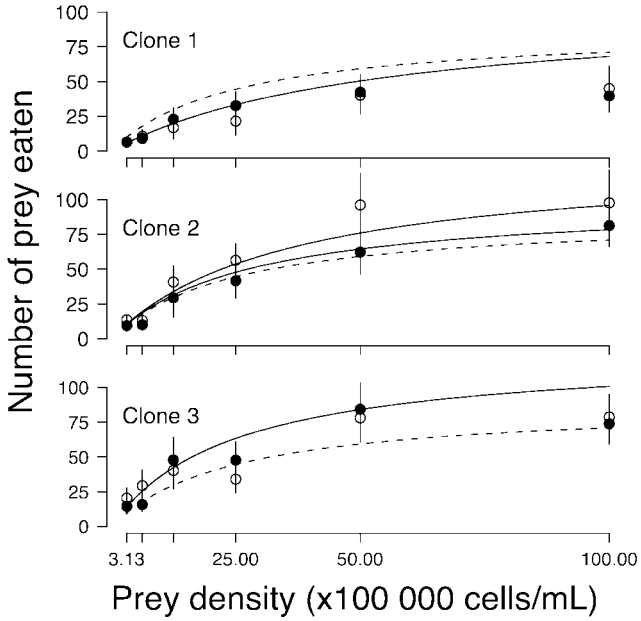

Fig. 3. Number of algae eaten as a function of algae density by three clones of *Euplotes* (±1 standard error). Solid lines are the most parsimonious models within each clone. In Clone 2, separate functions are required for undefended (○) versus defended (●) *Euplotes*. The dashed line is the best estimate when clone identity is ignored.

in how much this affected their ability to gather their own resources. This has implications for the stability of predator–prey systems with genetically different prey.

A model selection approach supported a Holling Type II functional response for the relationship between consumption rate and resource density. The induced defence increased handling time in *E. aediculatus* Clone 2, but did not detectably affect the functional response in either of the other clones. These differences in the functional response among clones are associated with greater morphological defence in *E. aediculatus* Clone 2 than in the other two clones. These data are thus consistent with the ideas that the reduced growth rate of induced *Euplotes* is partly mediated through altered foraging and that the presence of predators can indirectly affect consumer–resource interactions through trait-mediated mechanisms (Peacor and Werner, 2004). However, these effects will almost certainly vary within and among species with inducible defences.

Our experimental approach has several advantages. Our choice of a non-motile alga as resource removed any effect of resource motility on the feeding rate of *Euplotes*. Encounter rates with consumers are influenced by motility in resources (Gerritsen and Strickler, 1977), as is capture efficiency or handling time (Dolan and Coats, 1991). By taking advantage of the auto-fluorescence of chlorophyll, we avoided the need to label resource items artificially, which can alter cell surface properties in such a way that ingestion rates are decreased (Sanders, 1988). Counting the ingested cells microscopically allowed us to detect consumption of very low numbers of cells and account for variation among individual consumers. The short incubation time of *Euplotes* with algae eliminated complications due to cell proliferation or depletion of algae by *Euplotes*. Finally, the use of predator cues without live *Stenostomum*

Table 2. Model selection summary: Fit of Type II functional response models for induced and uninduced individuals in two clones of *Euplotes aediculatus* and one clone of *E. plumipes*

Model (shared parameters)	Log likelihood	*K*	AIC	ΔAIC	AIC weight
Euplotes aediculatus Clone 1 ($N = 99$)					
Type II (none)	−390.49	8	796.98	2.82	0.106
Type II (attack rate)	−390.63	7	795.26	1.10	0.250
Type II (handling time)	−390.80	7	795.61	1.45	0.210
Type II (all)	**−391.08**	**6**	**794.16**	**0**	**0.434**
Euplotes aediculatus Clone 2 ($N = 94$)					
Type II (none)	−445.17	8	906.35	1.36	0.312
Type II (attack rate)	**−445.49**	**7**	**904.99**	**0**	**0.634**
Type II (handling time)	−448.26	7	910.53	5.54	0.040
Type II (all)	−451.28	6	914.56	9.57	0.005
Euplotes plumipes Clone 3 ($N = 80$)					
Type II (none)	−353.34	8	722.67	2.39	0.135
Type II (attack rate)	−353.77	7	721.54	1.26	0.237
Type II (handling time)	−354.02	7	722.04	1.76	0.185
Type II (all)	**−354.14**	**6**	**720.28**	**0**	**0.445**

Note: All models were fitted using function nlme in program R under four conditions: (1) induced and uninduced individuals shared the same functional response parameters, shared only (2) attack rate or (3) handling time, or (4) they shared no parameters. Lower AIC values (in **bold** type) indicate the best model. *K* is the number of parameters in the model and *N* is the sample size.

predators eliminated predation effects such as loss of *Euplotes* individuals or their resources.

Ciliate feeding involves collecting food at the cytostome, ingestion via food vacuole formation, and digestion. In general, the rate of food vacuole formation is expected to be the limiting factor in the feeding process (Fenchel, 1986) and is the determinant of the maximum feeding rate (and therefore our estimate of handling time) in *Euplotes* (Dolan and Coats, 1991). Membrane required for food vacuoles is not synthesized *de novo* in *Euplotes* (Kloetzel, 1974), but is recycled from pre-existing vacuoles and transported back to the oral region via a dedicated system of microtubules (McKanna, 1973). The membrane supply mechanism thus limits ingestion at high prey densities, and is partly dependent on the rate of digestion. Morphological defence in *Euplotes* involves a restructuring of cytoskeletal elements in the development of defensive structures (Jerka-Dziadosz *et al.*, 1987). A reproductive delay in transforming cells (demographic cost of defence) is attributed to the diversion of microtubules and associated proteins, ready to be used for cell division, to the construction of these structures. If membrane for necessary cell functions is similarly in limited supply, morphological transformation may deplete membrane resources at the cost of food vacuole formation. The presence of lateral defence structures may also alter the morphology of the oral region in such a way that fluid currents generated by the adoral zone of membranelles are not as efficient for food collection. In either case, a resulting decrease in the feeding rate would indicate that defence influences handling time as we observed in Clone 2

Table 3. Parameter estimates: The attack rate (a) and handling time (b) from the best Type II functional response models for three *Euplotes* clones

	Estimate	Standard error
Euplotes aediculatus Clone 1		
a	1.947	0.906
b	0.00957	0.000699
1/*b*	104.5	
E. aediculatus Clone 2 (uninduced)		
a	3.663	1.212
b	0.00770	0.00189
1/*b*	129.9	
E. aediculatus Clone 2 (induced)		
a	3.663	1.212
b	0.01004	0.00189
1/*b*	99.6	
E. plumipes Clone 3		
a	5.108	2.298
b	0.00798	0.000470
1/*b*	125.3	
All clones combined		
a	3.575	0.671
b	0.0113	0.00121
1/*b*	88.4	

Note: Analyses were conducted for the number of *Chlorella vulgaris* eaten during 16 min of predation, for the three clones separately and with all clones combined. The asymptote (expected maximum predation rate in 16 min) is given as 1/*b*.

Euplotes aediculatus Clone 2 responded upon exposure to freezer-killed *Stenostomum* worms with a larger maximum width than the other two clones, an effect that coincided with an increased handling time in this clone. Even though the other two clones reached ~70% of the defence of Clone 2, we found no evidence that their functional response was affected by the defence, consistent with costs being an accelerating function of defence level. We previously found that even small levels of defence lower predation risk substantially, and gains with further increased defence levels are diminishing (Altwegg *et al.*, 2006). Taken together, these results can explain why *Euplotes* adjusts its defence so quickly and precisely to variation in predation risk (Duquette *et al.*, 2005). If initial costs were high, and defences only became functional once fully developed, one would expect the evolution of all-or-nothing defences rather than gradual ones.

Cost–benefit considerations are central to evolutionary models of inducible defence, and our results point to exciting new directions for future research. For example, the

Euplotes–Stenostomum system provides a powerful experimental model for asking questions about the relative costs and benefits of induced morphology and behaviour. As structural features, the development of morphological defences redirects resources from essential functions like growth and reproduction, whereas defensive behaviour tends to interfere with foraging and thereby inhibit growth through decreased food intake. It follows that the costs associated with each of these responses may influence defence expression differently, such that morphological defences are used when food is abundant and behavioural defences are used when it is not (Van Buskirk, 2000). Morphological defence in *Euplotes* increases with resources for a given predator density (Wiackowski and Staronska, 1999; Duquette *et al.*, 2005), but whether behavioural defence is favoured over morphological defence at low food densities has never been examined. It will be interesting to investigate the interplay of morphological and behavioural defences in future studies of *Euplotes*. Given enough clones with a weak correlation between behaviour and the level of morphological defence, it should be possible to separate their independent effects.

Even though *Euplotes* was clonal in our experiments, we still observed variation among cells in the level of induction (see also Duquette *et al.*, 2005). Variance in vulnerability of prey populations (Vos *et al.*, 2004a) is also expected to contribute to system stability. Direct tests of these effects have recently been accomplished in an algae–rotifer system (Verschoor *et al.*, 2004). The *Euplotes* system is a good candidate for additional tests of these fundamental questions in population and community dynamics.

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