

Ecological Effects of Inducible Antipredator Defense in the Ciliated Protist *Euplotes*

by

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Abstract

Inducible defenses alter the strength of interaction in food webs. Theoretical models that incorporate their effects are therefore critical for predicting community dynamics and stability. I examined ecological effects of an inducible morphological defense in a microbial model. I first investigated the effect of genotype, number of predators, and previous exposure to predators on the speed and maximum level of defense for eight clones in three species of the ciliate *Euplotes*. The effectiveness of defense depends on both of these aspects of defense induction; therefore these traits should evolve in concert. The speed and maximum level of induction varied among genotypes, showing that there is genetic variance for these traits and the potential for evolutionary change under selection. Higher predator densities led to more rapid induction and higher maximum levels of defense, but previous exposure to predators had no detectable effect on either of these traits. I then used a model selection approach to determine the shape of the functional response of clones that differed in their level of defense, and to estimate and compare the model parameters attack rate and handling time. Defense decreased the attack rate of *Euplotes* on *Chlorella vulgaris* algae in one highly defended clone, but did not affect the functional response in two less defended clones. My results demonstrate that *Euplotes* ciliates can precisely and rapidly adjust their morphological defense to the magnitude of predation risk in a way that varies among genotypes. This variation will lead to diversity in prey vulnerability to predators under natural conditions and translates to genetically-based differences in the foraging impact on resources of *Euplotes*. These estimates of ecological effects of induced defense in this system allow their inclusion in the development and testing of dynamic models. This in turn will inform our understanding of the influence of induced defenses and related trait-mediated indirect effects on community dynamics and stability.

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Chapter 1: General Introduction

Food webs are dynamic systems, within which spatial and temporal fluctuation in population numbers and multispecies interactions provide the context for evolutionary change. The development of predictive models that accurately describe the dynamics of these interactions is a current focus in community ecology (for recent reviews see Bolker et al. 2003, Werner and Peacor 2003). Traditional experiments have simplified communities in order to measure direct, density-mediated effects between species pairs. However, these relationships can change in response to other community members (Bender et al. 1984). These indirect effects can be transmitted through either the density or traits of mediating species (Abrams 1995) and can be larger than direct effects (Peacor and Werner 2004). Adaptive variation in behaviour is of particular interest as a mediator of indirect effects. Behavioural traits are expected to strongly affect the dynamics, structure and stability of communities in ways that may explain many inconsistencies between ecological theory and experimental data in food webs (Anholt 1997).

Although the relative roles of top-down and bottom-up regulation of dynamics are controversial, predator-prey relationships are the defining characteristic of food webs. Organisms are obliged to make a living through the exploitation of others, and the trade-offs of avoiding predation while maximizing growth and reproduction have presented strong selection for the evolution of defenses (Werner and Anholt 1993). Phenotypic plasticity, whereby different phenotypes can be expressed by a genotype in response to changing biotic or abiotic factors, offers a flexible approach for coping in a variable environment. It is not surprising that plastic defensive strategies have been adopted by many organisms. Inducible defenses, in contrast to constitutive (permanently expressed) defenses, are phenotypically plastic traits that are expressed only in the presence of potential biotic threats. They are widespread in nature and include responses to predators, herbivores, pathogens, parasites, and competitors. Chemical deterrents produced by grazed plants, adaptive foraging behaviour or life-history shifts in animals under risk of predation, and the vertebrate immune system are among the many

examples. Current theory regarding the evolution of induced defenses specifies four criteria for their maintenance (Tollrian and Harvell 1999a). Most importantly, selection imposed by the inducing agent must occasionally be strong but occur unpredictably. There also needs to be a reliable cue to indicate a potential threat and trigger the response. The defense must be effective, and it should incur costs which offset any advantage in the absence of threat. If there is no such trade-off or the threat is always present, constitutive defensive traits will evolve. This is supported by the general observation that induced responses are graded (e.g. Kusch 1993b, Van Buskirk and Arioli 2002), which presumably allows organisms to adjust defense to a level that is protective while minimizing associated costs (reviewed in Harvell 1990, Tollrian and Harvell 1999a).

Inducible antipredator defenses have been widely shown to be protective in the presence of predators. The nature and magnitude of defense-associated fitness costs are well known for some organisms, but are absent or difficult to measure in others (Tollrian and Harvell 1999b). Costs of induced defenses have important consequences for dynamics because, unlike direct predation mortality, they are suffered by the entire population and they are paid for the entire time that predators are present (Peacor and Werner 2004). Moreover, reduced population growth rates due to induced defenses do not translate into increased predator numbers (Ives and Dobson 1987), which results in fewer predators being supported by a given prey population. Investment in defense can be adjusted in response to changes in the density of predators, competitors, or resources, with subsequent changes in predation rates. If costs of defense are manifested in reduced food intake for defended prey, defenses can change population growth rates of all members in a tritrophic food chain. This is the case with antipredator behaviour that reduces activity levels under predation risk (e.g. Werner and Anholt 1993, Anholt et al. 2000, Brodin and Johansson 2004). If we are to predict realistic community dynamics, it may in many cases be necessary to incorporate inducible defenses and their natural variation into theoretical models.

Inducible defenses most directly affect the predator-prey interaction, which is characterized in population models by the predator's functional response i.e. the number of prey eaten as a function of prey density (Holling 1959). The most commonly fitted models to feeding rate data are the Holling Type I, II and III functional responses. These models are based on time allocation, in which the relationship between prey density and predation rate has two main components: the attack rate and handling time. The attack rate includes the rate of encounter, the probabilities of detection and attack, and the efficiency of attack. The handling time includes time spent attacking, eating and digesting prey. All predators and consumers are assumed to reach a plateau in their rate of food uptake. This maximum rate can be approached linearly (Type I), which indicates that a constant proportion of the available prey are consumed. Prey mortality is independent of density and thus cannot be controlled by predation. In a Type II response, the proportion of prey taken monotonically decreases at all prey densities to an asymptote determined by the handling time, indicating inverse density dependence where the prey population can escape control by the predator population. Type III responses have an initial increase in the proportion of prey taken as prey density increases, which results in a region of low prey density over which predators exert efficient control. At higher prey densities, a Type III curve converges on a Type II, with a monotonic decrease in predation up to the asymptote. The three models thus differ in their effect on community stability (May 1981).

Inducible prey defenses are expected to measurably influence the predator's functional response, increasing the handling time, decreasing the attack rate, or both (Jeschke and Tollrian 2000). A decrease in the attack rate translates into decreased uptake at low prey densities but has no effect at high prey densities, where the relative advantage of defense decreases. Increases in handling time due to defense indicate that defense does not prevent ingestion. This situation is not adaptive if attacks are always lethal, but it is when increased handling time is due to the time spent by predators in unsuccessful attacks. With morphological defense, it is possible that increases in handling time at low levels of defense allow more prey individuals to escape ingestion until a level of defense that decreases the attack rate is reached. Combined effects on the

handling time and attack rate may thus be more protective for some types of defense. These details of the predator's functional response when prey have inducible defenses are fundamental for modelling predator-prey population dynamics.

In theory, the community consequences of inducible defenses depend upon the details of the models (Ramos-Jiliberto 2003). For example, predator avoidance behaviour stabilized a predator-prey system when it was incorporated into Lotka-Volterra equations (Ives and Dobson 1987). Stability was observed because, if the defensive response is rapid, as in the case of behaviour and some morphological and physiological responses, then the net community effect is to dampen predator and prey density oscillations in this model. Conversely, models which incorporate time-lags between the onset of predation risk and expression of defense demonstrate a destabilizing effect of predator avoidance (Fryxell and Lundberg 1998, Luttbeg and Schmitz 2000). General stabilizing effects of prey refuges have been predicted under enrichment due to "donor controlled dynamics" (Abrams and Walters 1996). Variation in the timing and expression of defense can also result in heterogeneity in prey vulnerability (Vos et al. 2004a, 2004b). These factors tend to stabilize predator-prey dynamics in enriched bitrophic and tritrophic systems, a property that has been experimentally confirmed in a microbial system with rotifers and algae (Verschoor et al. 2004). In this experimental system, dynamics were affected by defenses in the basal trophic level (algae) but not by defenses in the middle level (herbivore). In contrast, Abrams and Matsuda (1997) found destabilizing effects of defenses in enriched systems. A Type II functional response was used in this and most other dynamic models. While these studies have begun to illuminate the role of inducible defenses in communities, they are still few.

It has been suggested that the influence of adaptive variation in behaviour and other inducible defenses on community dynamics should be assessed by experimentally manipulating phenotype (e.g. Anholt 1997). This is unfortunately not a simple process. Realistic dynamic models need to be constructed and parameterized by describing functional relationships (like the predator functional response) in short term experiments (Abrams 2001). The predictions of these models for dynamics then need to be tested in

long term experiments. Progress in this direction has been impeded by the lack of simple model systems of inducible defense.

I have been working on the development of a microbial defense model to assess the effects of inducible antipredator defense on ecological processes. These defenses have been observed in a number of ciliate protists (Wicklow 1997). By far the most widely studied are the defensive morphological transformations in species of the hypotrich genus *Euplotes* (Kuhlmann and Heckmann 1985). Several invertebrate and protist predators elicit a reaction in *Euplotes* through the release of waterborne polypeptide kairomones. The response involves a cytoskeletal reorganization (Jerka-Dziadosz et al. 1987) that results in a more spherical morphology relative to the usual flattened ovoid form. The most conspicuous effect is to increase the cell width (Figure 1) (Kuhlmann and Heckmann 1985). The dimensions of the transformed cell exceed the gape-limit of predators, protecting *Euplotes* from consumption (Kuhlmann and Heckmann 1994). Maximum induction is achieved within 24-36 hours and is reversed upon removal of predators or subculture of prey into fresh predator-free medium. The level of defense is directly related to the local density of predators and prey (Kusch 1993b, Wiackowski and Staronska 1999) and the availability of food (Wiackowski and Szkarlat 1996). Defensive transformation in *Euplotes octocarinatus* decreases population mortality rates by as much as 90-100% (Kuhlmann and Heckmann 1994). *Euplotes* can thus adapt defense to a level that is protective and thereby minimize associated demographic costs, which include a delay of cell division during transformation and reduced population growth rates in defended cells (Kusch and Kuhlmann 1994). Because *Euplotes* and many of their predators are readily maintained in culture and their defensive interactions are well characterized, they are ideal model organisms for evolutionary and ecological studies of inducible defenses and of phenotypic plasticity in general.

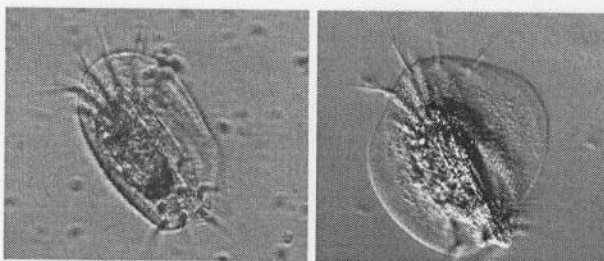


Figure 1-1: Cell morphology of undefended (left) and defended (right) *Euplotes octocarinatus*. The width of the undefended form is approximately 50 μm .

Defenses in *Euplotes* are induced by waterborne chemical cues (kairomones) released by their predators (Kuhlmann and Heckmann 1985). Kairomones have been isolated and characterized from the ciliate predator *Lembadion bullinum* (Kusch and Heckmann 1992), *Stenostomum sphagnetorum* (Kusch 1993c) and *Amoeba proteus* (Kusch 1993a). All are predator-specific polypeptides ranging in molecular weight from 10 to 31.5 kDa which act at extremely low concentrations (10^{-10} – 10^{-12} mol/L) (Kuhlmann et al. 1999). Induction of defence is dependent upon the concentration of kairomones, which increases with the number of predators. This indicates that *Euplotes* can assess predation risk and minimize costs of defense (Kuhlmann and Heckmann 1985). The predator-specific nature of kairomones allows *Euplotes* to deploy the appropriate defense level with different predators (Kusch 1995).

In summary, the *Euplotes* model system has many advantages. Protists and their predators have short generation times and are relatively easy to maintain in culture. Genetic markers (RAPD fingerprints) have been developed to distinguish between species and strains of *Euplotes* (Kusch and Heckmann 1996), an essential tool for tracking genotypes in long-term dynamic experiments. Also, inducing agents (kairomones) are transmitted at low concentrations through the aqueous medium to stimulate defense. Caged or otherwise inactivated predators can thus be used to separate direct and indirect effects of defense on population growth and persistence. Morphological defense is easy to measure and it can be correlated with behaviour because these ciliates express both types of defense.

In order to examine the role of adaptive defense in communities, levels of defense need to vary or be manipulated, preferably among closely related species or clones of a single genotype. There is natural variation in defense in *Euplotes*; some species do not display altered morphology or behaviour in the presence of some predators, while others show inter- and intraspecific variation in defense (Wiackowski et al. 2003). Genetically-based variation in defense has typically been measured at a single predator density. However, inducible defenses are phenotypically plastic traits and therefore must be measured over a variety of predator densities (the norm of reaction) in order to characterize variation (Underwood 2000). Given the theoretical importance of time lags in the effects of defense, the timing of its induction and experience of prior exposure to threat may also introduce variation. In Chapter 2, I compare these factors among species and clones of *Euplotes* exposed to cues from the turbellarian predator *Stenostomum virginianum* by measuring the time-course of defense induction over a range of predator densities and for ciliates that had been previously exposed to predator cues.

Many dynamically important relationships have already been measured for the *Euplotes* system, including that between predator density and defense level (Wiackowski and Staronska 1999, Wiackowski et al. 2003), which allows prediction of changing defense levels as predator densities fluctuate, and the prey population growth rate in relation to defense level (the cost of defense) (Kusch and Kuhlmann 1994). Prey vulnerability as a function of defense level (benefit of defense) and the functional response of predators on defended and undefended *Euplotes* have also recently been measured (Altwegg et al. unpublished). No data exist on whether the inducible defense affects the ability of *Euplotes* to exploit its own prey. If our ability to predict dynamic outcomes of community relationships requires measures of the trait-mediated indirect interactions (Werner and Anholt 1993), these data will be essential. Towards that end, in Chapter 3, I measure the functional response of *Euplotes* clones that differed in their level of morphological defense to *Stenostomum virginianum*.

Chapter 2. Factors affecting the expression of inducible defenses in *Euplotes*: genotype, predator density and experience.

Introduction

Inducible defenses directly affect food webs by changing the strength of interactions between prey and predators, and between competitors (Anholt and Werner 1999, Werner and Peacor 2003). Exactly what effect they have on community dynamics remains unclear. Inducible defenses have properties that tend to stabilize community dynamics (Ives and Dobson 1987, Vos et al. 2004a): their expression and effect is density dependent, and they create variation in prey vulnerability. Conversely, systems with predators that have saturating functional responses could be destabilized by prey defenses (Abrams and Matsuda 1997). Recent theory suggests that not only the maximum level of defense but also the timing of its induction can influence dynamics (Luttbegg and Schmitz 2000), and that longer time lags between exposure to predator cues and adoption of protective morphology can be maladaptive (Padilla and Adolph 1996, Gabriel 1999). The dynamic consequences of inducible defenses depend in part on the ability of prey to respond quickly to predation risk (Altwegg et al. 2004). If an inducible defense is reversible, previous encounters with predators may increase the speed and capacity to react in future encounters (e.g. Baldwin and Schmelz 1996). Two crucial aspects of inducible defenses are therefore the maximum level of induction and the speed with which prey induce upon contact with a predator cue. These two traits are likely to evolve in concert.

Inducible defenses are expected to evolve when predation risk is variable and unpredictable (Tollrian and Harvell 1999a). This assertion has been central in evolutionary studies of inducible responses, but in order for selection to lead to evolutionary change, there must be heritable variation in inducibility. Knowing the extent of variation in inducible defenses is therefore key for understanding both their evolution and their significance for community dynamics. Interclonal differences in antipredator responses, for example, suggest genetic variation and may lead to

evolutionary change under different predation regimes. Of the few studies that demonstrate genetic variation for inducible defenses in plants (Zangerl and Berenbaum 1990, van Dam and Vrieling 1994, English-Loeb et al. 1998, Underwood 2000, Underwood et al. 2000), animals (Parejko and Dodson 1991, Spitze 1992, Harvell 1998), and protists (Wiackowski et al. 2003), none have considered the speed and maximum level of defense simultaneously. Most of these studies examine two levels of induction, defended vs. undefended. Few have compared the entire reaction norm among clones (Parejko and Dodson 1991, Underwood 2000).

This study examines variation in the speed and maximum level of induction among species and clones of *Euplotes* ciliates. In response to kairomone signals from various predators, *Euplotes* transform into a more spherical morphology relative to the usual flattened ovoid form. This change involves a cytoskeletal reorganization (Jerka-Dziadosz et al. 1987) and leads to a conspicuous increase in width (Kuhlmann and Heckmann 1985). The transformed cell dimensions exceed the gape-limit of predators, protecting *Euplotes* from consumption (Kuhlmann and Heckmann 1994). In order to compare the extent and timing of defense among genotypes, I performed two separate experiments. In the first, referred to as a time-course experiment, I exposed *Euplotes* clones to various densities of the predatory turbellarian *Stenostomum virginianum* and compared their reactions over time. In the second experiment, referred to as a memory experiment, I examined the effect of previous encounters with predators on the speed and maximum level of induction in several clones of *Euplotes*.

Measuring the speed of induction is not straightforward if it is developed at a non-linear rate, as is the case with morphological defense in *Euplotes*. I therefore examined two measures of the speed of induction: i) the time needed to reach half of the maximum induction, and ii) the width reached six hours after exposure to predators. The first measure is related to the amount of time spent actively transforming, a developmental stage at which cell division and therefore population growth rates are strongly decelerated (Kusch and Kuhlmann 1994). This measure may not predict very well how rapidly the defense becomes effective against gape limited predators, because this depends more on

the absolute cell width. I therefore considered a second, absolute measure of induction speed, the cell width reached after six hours, when all genotypes were actively transforming.

Materials and Methods

Model Organisms and Culture Procedures

I examined inducible defense in several species of *Euplotes* responding to their turbellarian predator *Stenostomum virginianum* Nuttycombe 1931. *Euplotes* prey were kindly provided by K. Wiackowski (Jagiellonian University, Krakow, Poland). *Euplotes octocarinatus* Carter 1972 clones were descendants of single cells, isolated from either a mixed aquarium population, or from a natural pond (Wiackowski et al. 2003). *Euplotes aediculatus* Pierson 1943 and *E. plumipes* Stokes 1884 were obtained from the same laboratory but are of unknown origin. I maintained all *Euplotes* isolates 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., Canada). Inoculates of *Bacillus cereus* (Boreal Laboratories, St. Catherines, ON, CAN) in tryptic soy broth (Difco Laboratories, Detroit, MI, USA) served as food for the ciliates. The predatory flatworm *Stenostomum virginianum* (Rhabdocoela: Turbellaria) was isolated from sediments of a freshwater pond on the University of Victoria campus. I used either live or freezer-killed *Stenostomum* worms (~350 worms/mL, stored in 1.5 mL Eppendorf tubes, at -4°C) to induce prey defenses in all experiments. *Euplotes* subcultures were transferred into new medium every three to five weeks.

Time Course of Defense

The objectives of this experiment were (1) to characterize the time-course of morphological transformation in *Euplotes* at several predator densities and (2) to establish interspecies and interclonal variation in the time-course of defense. To examine these objectives, I exposed eight clones (three clones of *Euplotes aediculatus*, four clones of *Euplotes octocarinatus*, and one clone of *Euplotes plumipes*) to five densities of live *Stenostomum* predators and measured their cell width over the time of morphological transformation, which typically takes place over 24 to 36 hours.

On each of three different dates, I set up two complete randomized blocks of all experimental treatments in 24-well culture plates (Costar, Corning) in 1.0 mL total liquid volume. Some of the replicates experienced high mortality associated with the transfer into the new containers, and I therefore ended up with 2 to 6 replicates in total for each treatment. Live *Stenostomum* predators (2, 4, 8, or 16) were counted from dense cultures into wells in 500 μ L of culture fluid. The addition of live predators did not lead to significant size-selective predation, as the observed size changes of the ciliates far exceeded the variation in their initial population. I added 500 μ L of NAYA water to zero-predator control wells. *Euplotes* cells were transferred from well-established cultures and pooled in fresh bacteria-inoculated medium, and then 150 cells were counted into experimental wells in 500 μ L of medium. The time of addition of each *Euplotes* clone was time zero for that set of replicates.

The time-course of defense was examined at different predator levels by photographing each well at 2, 6, 12, 18, 24, and 36 hours post-induction. I scanned the bottom of each well systematically with an inverted microscope (Leica DM-IRB Wetzlar, Germany) at 100x magnification and captured images through a Cohu CCD camera (San Diego, USA) of the first ten individuals encountered using Image Pro Plus 4.5 image analysis software (Media Cybernetics, Silver Spring, USA), which was also used to measure the maximum width of each cell.

When exposed to predators, *Euplotes* cells respond quickly with a large initial increase in width, then asymptotically reach their final maximum width. In order to best account for this time course, a third order polynomial was fitted to the median width values of the cells measured over time. I used median rather than mean width to reduce the influence of extreme cells. A separate equation was fitted to the data from each experimental replicate, and three values were obtained from these equations for use in further analyses. The first value was the maximum of the fitted curve, which represents a measure of the maximum level of induction (width) reached by each clone at each predator level. The other two values were measures of the speed of induction: the width reached after six hours, and the time until half of the maximum cell width was reached. The variation in defense was then examined between species, and between clones within species, by comparing these estimates using nested analysis of variance. These analyses were performed using procedure aov in program R 1.8.1 (Ihaka and Gentleman 1996).

Memory in Defense

The objectives of my second experiment were to (1) determine whether past exposure to predators has any effect on the speed and maximum level of induction of the defended form in *Euplotes* (memory effect), and (2) to establish potential variation in memory between species and clones. I investigated these objectives using an experimental design similar to that of Baldwin and Schmelz (1996). This design consists of three episodes during which *Euplotes* were either exposed to a predator cue (1) or to predator-free control medium (0), depending on the treatment. The experimental treatments consisted of 1 (001), 2 (011, 101) or 3 (111) exposures of *Euplotes* to frozen *Stenostomum* worms. Between induction episodes, the predator kairomone cues were diluted out for 2-3 weeks through the removal and replacement of culture medium, and the cells regained their undefended form. During the final induction episode, all experimental units received the predation cue, and I compared the speed and amount of induction of prey that had been exposed to the different predator treatments. The predation cue consisted of frozen *Stenostomum* worms (~350/mL). The experimental units were individual wells in 24-well culture plates (Costar, Corning) with a volume of

750 μL and one-half sterilized wheat grain to provide nutrients to support bacterial growth. All wells were stocked with 100 *Euplotes* cells. I arbitrarily chose two clones of *E. aediculatus*, two clones of *E. octocarinatus*, and one clone of *E. plumipes* for this comparison, and replicated the whole experiment six times in total, distributed over two temporal blocks. Treatment 001, where *Euplotes* was induced only once in the last episode, was doubly replicated to increase the power of the comparison for naïve cells versus the treatments with prior predator exposure. The long duration of this experiment required replacing 500 μL of culture fluid from each experimental well with 500 μL of clean NAYA water every 2-3 days during the relaxation period. This served to maintain the initial density of cells as closely as possible and to minimize bacterial overgrowth in the wells.

Six and 36 hours after the final exposure to predator cues, I measured the width of 12 randomly chosen cells in each well using the methods described for the time-course experiment, except that before the second measurement, all of the wells were preserved with acid Lugol's solution to stop the experiment and to facilitate data collection. Preservation may affect the cell shape of ciliates (Chaput and Carrias 2002), although I did not observe any obvious changes in shape when taking the measurements immediately after preservation. I nevertheless avoid comparing these measurements with ones of live cells. I also obtained width measurements of live cells before and 48 hours after each induction episode to verify the expected response to the treatments. I used cell width 6 and 36 hours after the final induction as measures of the speed and maximum level of induction. These data were analyzed using mixed effects models in the procedure lme of the program R 1.8.1 (Ihaka and Gentleman 1996), treating block and well as nested random effects, and testing the treatment effects at the between-well level to avoid pseudo-replication.

Results

Time Course of Defense

In this experiment, I characterized the time course of morphological antipredator defense in clones of three species of the hypotrich ciliate *Euplotes* exposed to several predator densities. I then examined variation between clones and species in speed and maximum level of defense induction.

Euplotes responded to predators with a dose-dependent increase in width that was measurable within six hours and completed in 12-18 hours (Figure 2-1). *Euplotes plumipes* and *E. aediculatus* 3 both had a very fast initial response to predators, evident as an increase in width in predator treatments relative to the control at two hours post-induction. Separation between the zero predator control and predator treatments in the other species and clones was not as obvious until about 6 hours post induction. Widths in the zero predator controls remained near baseline values for all clones, with the exception of *Euplotes octocarinatus* 4, which increased slightly over the course of the experiment, and *E. aediculatus* 3, which decreased.

I measured the speed of induction in two different ways: (i) as the time needed to reach half maximum induction, and (ii) as the width six hours after exposure to predators. (i) The time needed to reach half maximum induction was not significantly affected by the number of predators present in each well, and I found no overall difference among species in their speed of reaction to predators (Table 2-1). However, clones within species did differ in the speed of induction, variation which is primarily among clones of *E. octocarinatus* (Figure 2-2a). Clones 4 and 6 responded particularly rapidly, reaching half maximum induction within six hours, almost two times more quickly than clone 5 at ten hours and three times more quickly than clone 7 at 16 hours. (ii) At six hours, cells were on average 17.67 μm wider when exposed to predators than in the predator-free treatment (Table 2-2, Predator presence), and their width increased with predator density by 0.23 μm per predator (Table 2-2, Number of predators). I found significant

differences among species and clones in the width at six hours (Figure 2-2b, Table 2-2). As clones and species differed in their undefended cell width, the information on whether they differed in their reaction to predators is contained in the interaction terms between predator treatment and clone/species. The only significant effect was the variation among clones in their overall reaction to predators (Table 2-2), and this result is thus identical to the one for time to half maximum induction (i, above).

Table 2-1. ANOVA summary for the effects of predator number, species, and clone on the speed of induction (time to half maximum induction). This analysis nested clone within species and only included treatments with predators present.

	df	Sum of Squares	Mean Square	<i>F</i>	<i>p</i>
Predator number	1	0.69	0.69	0.03	>0.8
Species	2	255.85	127.92	1.03	>0.4
Clone(Species)	5	619.62	123.92	5.48	0.0001
Block	2	91.57	45.79		
Residuals	132	2982.89	22.60		

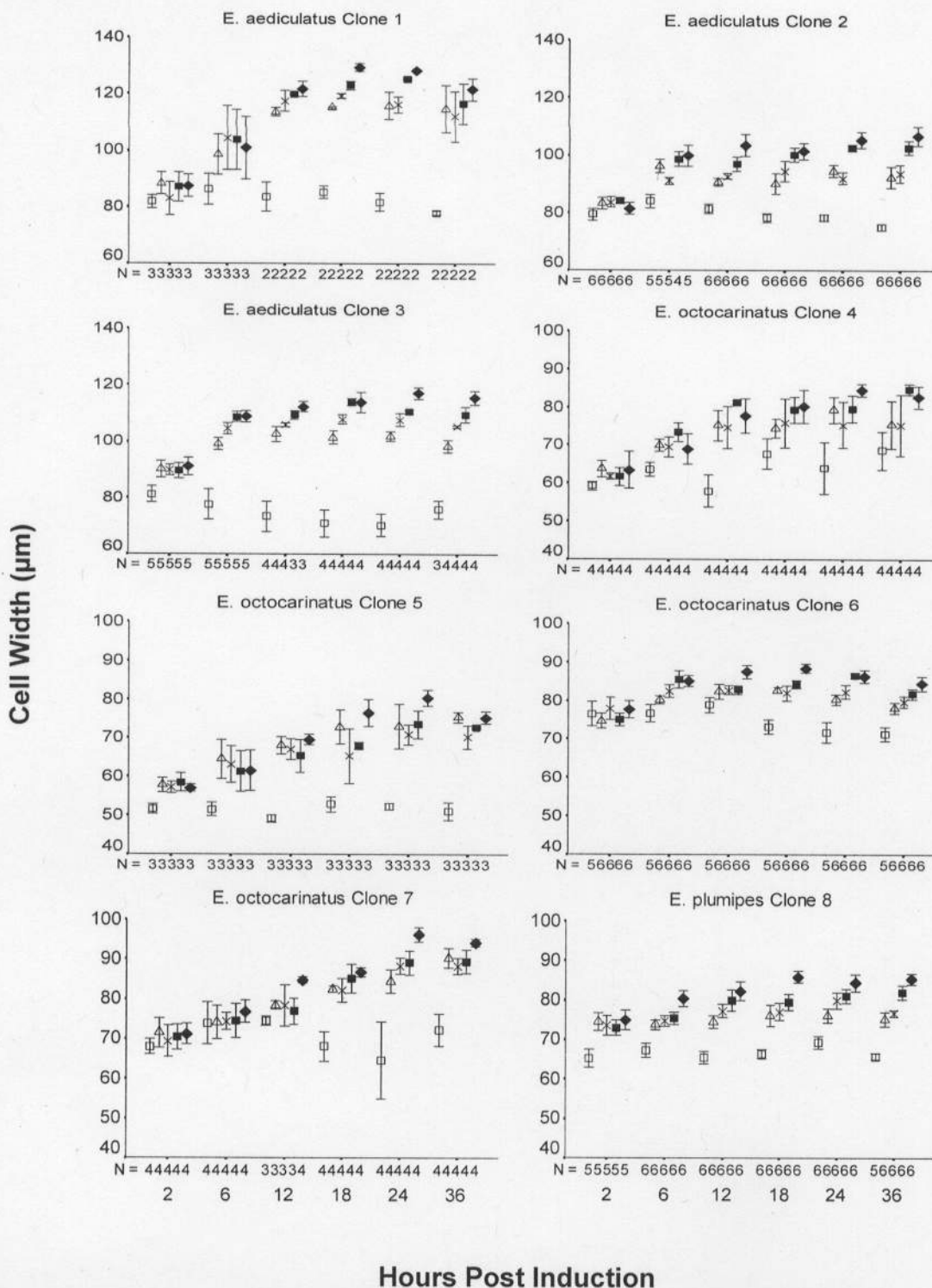


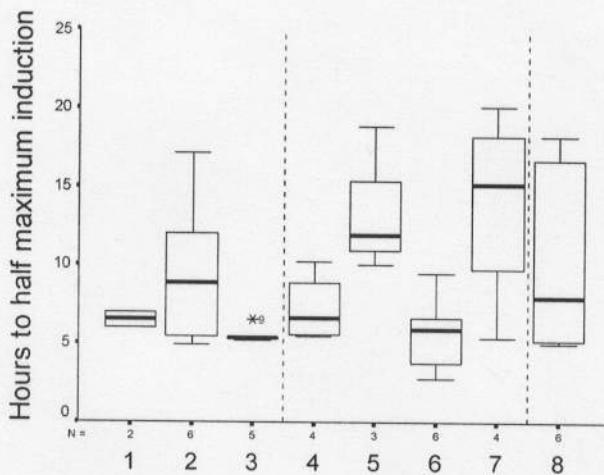
Figure 2-1. Time-course of a defensive morphological reaction norm in eight genotypes of Euplotes. The treatments consisted of: \square 0, \triangle 2, \times 4, \blacksquare 8, \blacklozenge 16 predators/unit. Data represent the means (\pm Standard Error) of two to six experimental replicates per treatment (N), using the median width of ten cells per replicate.

Table 2-2. ANOVA summary for effects on cell width six hours after exposure to predators. Predator presence (PP) contrasts the predator-free treatment with the mean of the others, and its significance shows that *Euplotes* already reacted at this time. The number of predators (NP) is treated as a linear covariate, and its significance shows that *Euplotes* reacts more rapidly when exposed to higher predator densities. The factors species, and clones (nested within species) account for overall differences among the genotypes. The information whether the genotypes differed in their reaction to predators is contained in the interaction terms.

	df	Sum of Squares	Mean Square	F	p
Predator presence (PP)	1	2802.70	2802.70	154.24	<0.001
Number of predator (NP)	1	208.60	208.60	11.48	0.001
Species	2	1988.30	994.20	0.82	0.493
Block	2	18258.80	9129.40		
PP X species	2	804.20	402.10	3.16	0.130
NP X species	2	85.10	42.60	3.80	0.099
Clone(Species)	5	6086.70	1217.30	67.00	<0.001
PP X Clone(Species)	5	636.00	127.20	7.00	<0.001
NP X Clone(Species)	5	56.00	11.20	0.62	0.688
Residuals	152	2761.90	18.20		

I found significant differences among species and clones in the maximum width obtained upon exposure to predators (Table 2-3, Figure 2-3). Again, the interaction terms between predator treatment and clone/species show whether the genotypes differed in their reaction to predators. The species differed in their reaction to predator density, and the clones within species differed in their overall reaction to predator presence (Table 2-3).

(a)



(b)

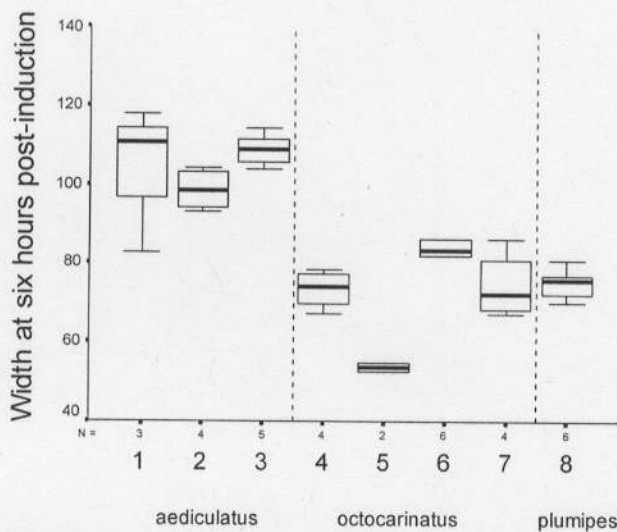


Figure 2-2. Interspecies and interclonal variation in the speed of induction of *Euplotes* exposed to eight predatory *Stenostomum* worms. Induction speed was measured as the time to reach half maximum induction (a) and the cell width 6 h after exposure to predators (b). Data show the median values and extend from the maximum to the minimum estimates.

Euplotes octocarinatus clone 5 did not react as strongly as the other clones within that species (Figure 2-3). Clones 4 and 6 reached similar maxima, though clone 6 was larger initially and therefore had a smaller overall gain in width. Clone 7 achieved the greatest width of the *E. octocarinatus* clones, and spent more time transforming than the others. The three clones of *E. aediculatus* also attained significantly different maximum widths, and *E. plumipes* reacted similarly to *E. octocarinatus* (Figure 2-3).

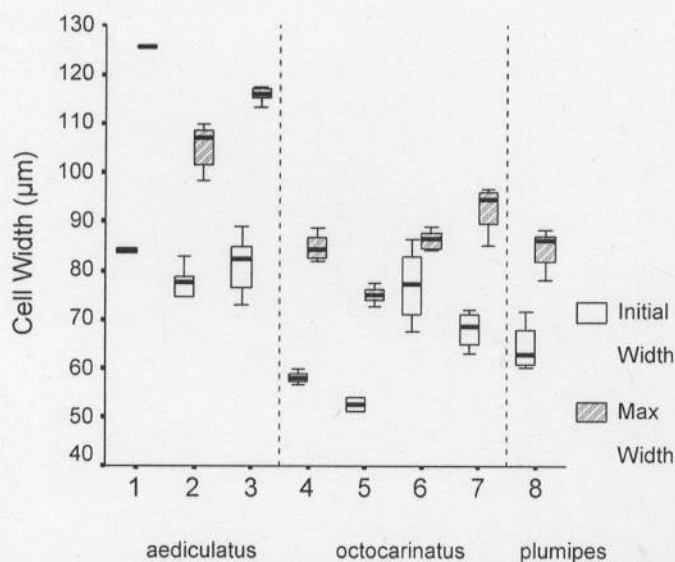


Figure 2-3. Interspecies and interclonal variation in maximum induction in *Euplotes* after exposure to eight predatory *Stenostomum* worms. Initial widths are those expressed in predator-free control treatments

Table 2-3. ANOVA summary table for the effect of predator presence (PP), number of predators (NP), species and clones on maximum cell width. Clones were nested within species; see Table 1-2 for further details.

	Df	Sum of Squares	Mean Square	<i>F</i>	<i>P</i>
Predator presence (PP)	1	7998.30	7998.30	373.67	<0.001
Number of predators (NP)	1	1592.60	1592.60	74.41	<0.001
Species	2	17479.20	8739.60	10.27	<0.02
Block	2	3125.00	1562.50		
PP X species	2	1031.30	515.70	2.95	0.14
NP X species	2	240.80	120.40	18.52	<0.01
Clone(Species)	5	4254.40	850.90	39.75	<0.001
PP X Clone(Species)	5	873.50	174.70	8.16	<0.001
NP X Clone(Species)	5	32.60	6.50	0.30	0.91
Residuals	152	3253.50	21.40		

Memory in Defense

Here, I exposed two clones of *E. aediculatus*, two clones of *E. octocarinatus*, and one clone of *E. plumipes* to predator cues 1-3 times, and asked whether previous exposure leads to more rapid induction or a higher maximum level of induction at the final episode. I found no effect of previous induction on cell width six hours (my measure of speed of induction) or 36 hours (maximum level of induction) after the final exposure to the predator cue (Fig. 2-4, $F_{3,55} = 1.01$, $P = 0.39$ and $F_{3,94} = 1.27$, $P = 0.29$), and thus no evidence for a memory effect. These results remained the same when the number of previous exposures was treated as a covariate, or when the treatment with no previous exposure was contrasted with all of the other treatments (all $P > 0.09$). The effect that came closest to statistical significance had the opposite sign than expected if there was a memory effect (effect of number of exposures on cell width at six hours: -0.96 , $SE = 3.02$). There was also no significant difference among genotypes in a potential memory effect regardless of how I analyzed the number of exposures (interaction between treatment and genotype: all $P > 0.07$).

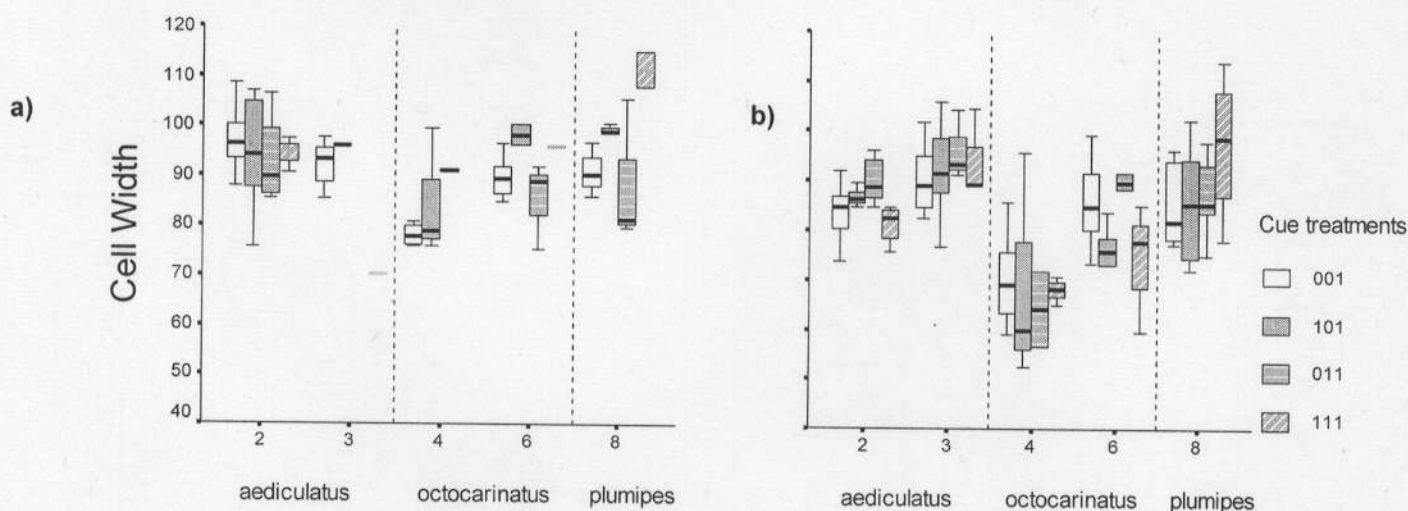


Figure 2-4. Effect of prior induction on morphological antipredator defense in *Euplotes* (a) 6 h and (b) 36 h after exposure to predator cues. *Euplotes* were exposed to a predator cue (1) or control medium (0) during three induction episodes. Data shown are for measurements taken during the final episode when all treatments were exposed to the predator cue.

Discussion

In two experiments, I examined the effects of predator density and previous exposure to predators on the speed and maximum level of induction of a morphological defense in eight genotypes of the ciliate *Euplotes*. While *Euplotes* precisely adjusted their level of defense to predator density, there was no detectable effect of previous predator exposure on either the speed or maximum level of induction. The genotypes strongly differed in their speed of reaction, in the maximum level of defense, and in their reaction to predator density. The variation between clones was often larger than the variation between species.

My experiment examining the time course of induction showed that *Euplotes* can react quickly and precisely to different levels of predation risk. This is consistent with earlier studies on *Euplotes* (Kuhlmann and Heckmann 1985, Kusch 1993b, 1995, Wiackowski and Szkarlat 1996, Kuhlmann et al. 1999) and other organisms (Anholt et al. 2000, Underwood 2000, Van Buskirk and Arioli 2002, Relyea 2004) showing that the level of defence increases with predator or herbivore density. Most genotypes in my experiment reacted strongly to the lowest predator density and then increased their defense only by smaller amounts when exposed to higher predator densities (Fig. 2-1). This suggests that the ecological effects of this inducible defense are strongest when predator densities vary around very low values, because at high predator densities all prey tend to be well protected. On the other hand, the defensive morphology may only be effective when expressed at a high level and further small increases in defense level may translate into large differences in vulnerability. Kusch (1995) examined the relationship between defense level in *Euplotes* and predation risk by *Stenostomum* and found that this morphological defense becomes effective at about 85 μ m cell width, which *E. octocarinatus* and *E. plumipes* reached only at high predator densities in this experiment. In contrast, it has been found that low levels of defense are very effective and higher levels of defence do not further decrease vulnerability much (Altwegg et al. unpublished). It depends in part on the size of these turbellarian predators.

I found variation between genotypes for all aspects of this morphological defense: speed of induction, maximum level of induction, and the relationship between predator density and defense level. This suggests that there is genetic variation and thus the potential for complex evolutionary change in this trait. The speed of induction is important if predation risk fluctuates rapidly because only a prompt reaction can ensure that the phenotype matches the environment (Padilla and Adolph 1996, Gabriel 1999). West-Eberhard (1989) proposed that the lability of a trait may influence the evolution of plasticity in that trait. Yet, the speed of induction for a defense has rarely been examined (but see Van Buskirk 2002), and I know of no study that has examined genetic variation in this trait. Variation in the maximum level of induction, on the other hand, has previously been found in *Euplotes* (Wiackowski et al. 2003), some animals (Parejko and Dodson 1991, Spitze 1992, Harvell 1998), and plants (Zangerl and Berenbaum 1990, van Dam and Vrieling 1994, English-Loeb et al. 1998, Underwood et al. 2000). I found that the variation between clones was generally larger than the variation between species, which highlights the need to consider several genotypes when comparing inducible defences among species.

The results of the second experiment gave no indication that previous predator exposure affects the speed or maximum induction in *Euplotes*. The most familiar case of such a memory effect is the vertebrate immune system, which is an inducible defense against pathogens. Sessile marine invertebrates can also show stronger aggressive responses against competitors they are familiar with (reviewed in Harvell 1990). Other than this, memory effects in inducible antipredator defenses have apparently been investigated only in one case: the tobacco plant *Nicotiana sylvestris* produces nicotine upon attack by herbivores and does so more rapidly if it was previously attacked (Baldwin and Schmelz 1996). *Euplotes* may already maximize the speed of induction, and in contrast to chemical defense or immune systems, it may not be possible to store components of this morphological defense over long periods of time. In my experiment, two to three weeks elapsed between inductions. This corresponds to 10 to 20 cell cycles, which may have been too long to find a memory effect. Still, this defense is maintained during cell division and I wanted to be sure that *Euplotes* had completely lost their

previous induction. If a partially induced *Euplotes* cell re-encounters a predator, I expect it to regain full induction quickly, strictly because the defense is already partially expressed. In an earlier experiment, it was found that *Euplotes* continually adjust their defense and this led to changes in the defense level on a similar time scale as the population dynamics of prey and predators (Altwegg et al. 2004).

In conclusion, these experiments show that predator density and genotype affect the expression of inducible defenses in *Euplotes*. This is important for understanding both the evolution of inducible defenses and their effect on community dynamics. My results demonstrate that *Euplotes* can precisely and quickly adjust defense to the current risk of predation and that there is scope for complex evolutionary change in this trait. They also suggest that there is always variability in defence and thus vulnerability within populations of *Euplotes* in natural situations where predator densities fluctuate and several genotypes coexist. This is a crucial factor leading to more stable community dynamics and higher equilibrium population densities in the prey (Leibold 1989, Bohannan and Lenski 1999, Vos et al. 2004a).

Chapter 3. Inducible Antipredator Defense affects the Functional Response of the Ciliate *Euplotes*

Introduction

Prey defenses that are induced by cues associated with predation risk are widespread in natural systems (Lima and Dill 1990, Lima 1998, Tollrian and Harvell 1999a). They have been repeatedly shown to decrease predation mortality and therefore affect vital rates of predators and prey. In order to accurately describe the dynamic behaviour of natural food webs, there is a clear need to incorporate inducible defenses into theoretical predator-prey models and to experimentally test their predictions. While there are many well-characterized natural systems with inducible defenses, little research has been directed at their influence on functional characteristics of dynamic relationships. Measurement of the effect of inducible defenses 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 defenses, and models that include them focus on the defended prey and its predator. There is evidence, however, that expression of defense can alter the relationship between the inducible prey and its own resources (Ramcharan et al. 1992, Grunbaum 1997, Anholt and Werner 1999, Tollrian and Dodson 1999, Trussell et al. 2003).

Typically, defended prey have lower rates of somatic or population growth. This is predicted by theory (Rhoades 1979, Harvell 1986, Lively 1986a, Harvell 1990, Clark and Harvell 1992, Harvell and Tollrian 1999), which argues that without such fitness costs, evolution selects for constitutive defenses. Costs have been repeatedly demonstrated in plants (e.g. Baldwin et al. 1990, Zangerl et al. 1997, Zavala et al. 2004), cladocerans (e.g. Havel and Dodson 1987, Riessen and Sprules 1990, Tollrian 1995, Boeing et al. 2005), bryozoans (Harvell 1992, Grunbaum 1997), ciliates (Kusch and Kuhlmann 1994), barnacles (Lively 1986b) amphibians (Anholt and Werner 1999, Van

Buskirk 2000) and fish (Pettersson and Bronmark 1997). A reduced rate at which defended prey can consume their own food, caused by changes in morphology or behaviour, may be one such cost.

The functional response, which describes the rate of prey intake as a function of prey density, is a fundamental component of predator-prey models. The nature of the functional response relates to characteristics of the predator-prey relationship and affects its inherent stability. The distinction between Type II and III responses (Holling 1959) is particularly significant; Type III responses are stabilizing over a range of prey densities because the proportion of prey consumed initially increases with prey density, while Type II responses are destabilizing because the proportion of prey consumed declines with density for all prey densities. Measurement of differences in functional response model parameters between defended and undefended prey reflects ecological costs of defense and allows for the integration of these costs into predator-prey models. This approach has been used to demonstrate that the benefits of a predator-induced morphology are due to an increased handling time in a predator with a Type III functional response (Altwegg et al. unpublished manuscript). While studies regularly show that defended prey are less likely to die of predation, this is one of few experiments to determine whether the reduction in predation is due to changes in the likelihood of attack or the time spent handling prey (Iyer and Rao 1996, Mohr and Adrian 2000, Chang and Hanazato 2005).

I extend this work in the present study, by further examining the relationship between consumption rate and prey density in a microbial model system of inducible defense. The ciliate *Euplotes* has an interesting set of inducible responses that are initiated within a few hours of exposure to predators like the turbellarian worm *Stenostomum* (Kuhlmann and Heckmann 1985). Ordinarily a flattened ovoid cell, induction in *Euplotes* leads to the growth of primarily lateral structures, which increase the cell width above the predator's gape limit and inhibit ingestion (Kuhlmann and Heckmann 1994). This process requires considerable energy and protein for cytoskeletal

microtubules (Jerka-Dziadosz et al. 1987), and costs *Euplotes* a delay in cell division. Reproduction 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. The trade-off between predation risk and growth is generally expected to result in reduced foraging in the presence of predators (Werner and Anholt 1993). While predator avoidance behaviour has been observed in *Euplotes* (Kuhlmann 1994), the costs associated with this form of defense are not known.

Euplotes ciliates are motile filter feeders. Cells extract particles from the aqueous medium using the adoral zone of membranelles (AZM), a highly specialized oral ciliature that creates fluid currents to direct prey 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. Grazing rates for microzooplankton have been estimated using the change in prey concentration over hours or days of incubation with predators (Landry and Hassett 1982). These assays require relatively high prey densities, which appreciably change during the experiment. Direct counting of fluorescently labelled prey (Sherr et al. 1987) has the advantage that short incubation times (minutes) can be used, which is necessary for functional response measurements because prey depletion is so slight it can be ignored. Prey can be live-stained, which has no effect on their motility, 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 the autofluorescence of chlorophyll (e.g. Premke and Arndt 2000).

Here, I directly counted ingested algae within defended and undefended *Euplotes* cells of three clones in two species allowed to forage at a range of algae concentrations. I then considered all three of the basic functional responses originally proposed by Holling (1959), to determine which one best described the effect of prey density on the predation rate and whether the expression of inducible defense affected this relationship.

Materials and Methods

Model Organisms and Culture Procedures

I compared the functional response of clones within two species of *Euplotes* 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). I used the unicellular green algae *Chlorella vulgaris* (University of Toronto Culture Collection, Strain 266) as prey. 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 μ L of a mid-log phase liquid culture of *Bacillus cereus* (Boreal Laboratories, St. Catharine's, ON, CAN) 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 (BBM) under a constant light source (Philips F20T12/ww 20 watt full spectrum fluorescent tubes). Filtered air (0.22 μ m) was bubbled through the cultures to keep the algae and nutrients well distributed.

I 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, CAN) and sterilized wheat grains in 100 x 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. In order to eliminate the effects of predation in this experiment, I used freezer-killed *Stenostomum* rather than live worms to induce morphological changes in *Euplotes* (Altwegg et al. 2004).

Functional Response of Euplotes

I was able to examine the functional response without concern for prey depletion, by using the autofluorescence of chlorophyll to count directly the number of *Chlorella vulgaris* cells ingested by *Euplotes* cells over a range of prey concentrations in a short-term experiment. Defended and undefended populations of two clones of *E. aediculatus* and one clone of *E. plumipes* were compared.

I set up completely randomized blocks of experimental replicates in 24-well tissue culture plates (Costar, Corning). There were nine blocks, each set up on different dates. *Euplotes* cells were resuspended from well-established cultures into fresh sterile medium to wash away any bacteria and 60 cells were counted into experimental wells in 200 μ L of fluid. Either 200 μ L of freezer-killed worms (200 worms/mL, -4° C) (defended treatments) or 200 μ L of Naya water (control/undefended treatments) were added to make a final volume of 600 μ L. The experimental plates were then incubated at room temperature for 24 hours to allow induction of defensive morphology to take place in the defended treatment wells. I took live photomicrographs of randomly sampled *Euplotes* cells from 5 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. I measured the maximum cell width from the photographs to confirm induction of defensive morphology and compare the level of defense among the three clones.

To assay the predation rate, I added 200 μL of *Chlorella vulgaris* culture to each experimental well. The final concentrations of algal cells ranged from 1×10^7 cells/mL to 3.13×10^5 cells/mL over a 1/2 dilution series, for a total of six prey levels. I thus 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 sixteen minutes, and the experiment was stopped by adding 6 μL of 20x diluted alkaline Lugol's solution. This was followed by 12 μL of borate-buffered formalin to preserve the cells and 7.2 μL of 4x diluted 3% sodium thiosulfate to clear any iodine staining from the Lugol's solution, which masks chlorophyll fluorescence (Sherr et al. 1987). To prepare samples for epifluorescence microscopy, I transferred all of the preserved *Euplotes* cells in each well into 1 mL of sterile NAYA water by pipette while observing through a stereomicroscope (Leica M8). I 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. I 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. I was then able to count the number of algae that had been eaten in the sixteen minutes of the experiment, as they were clearly visible within the cells (Figure 2.1). 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.

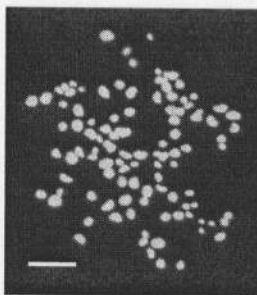


Figure 3-1. Visualization of ingested algae inside *Euplotes* during epifluorescence microscopy. Image shown is *E. aediculatus* Clone 1. Cells were fixed with alkaline Lugol's iodine solution and borate-buffered formalin after sixteen minutes of incubation with *Chlorella vulgaris* algae. Visualization at 400x (Zeiss epifluor 40x objective). Scale Bar = 20 μ m.

Statistical Methods

I wanted to fit simple representations of Holling Type I, II, and III functional response models to the data, and look for differences in model parameters among clones and between defended and undefended *Euplotes*. The following equations were used to examine the effect of prey density (X) on the number of prey eaten (per *Euplotes* cell in sixteen minutes of grazing), (y):

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

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

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

Equation (1) is a simple linear regression model, in which separate estimates of the slope and/or intercept were fitted for the two treatments to test for the effect of defense.

Equation (2) is a hyperbolic function, where c is the slope of the curve at the origin (determined by the predator's attack rate) and $1/d$ is the asymptote (determined by d , the predator's handling time). Equation (3) is a sigmoid modification of Model 2 where the attack rate increases with prey density. Separate estimates were also used for attack rate and/or handling time in defended and undefended treatments when fitting Type II and

Type III models. 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. These forms for the functional response are appropriate for this experiment because the depletion of algal prey relative to initial prey numbers was essentially zero over the sixteen minutes of grazing, so the assumption of constant prey density was met (Juliano 2001). Versions of the three functional response equations were fitted to the data from each clone separately by maximum likelihood using function `nls` in program R v2.01a (Ihaka and Gentleman 1996). Data from all three clones were also combined for an analysis to test whether the clones differed in their functional responses.

Comparison of the models was based on Akaike's Information Criterion (AIC), where the lower AIC values in a model set indicate those that are the best 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 evidence ratios, to determine the relative strength of support for one model over another (Burnham and Anderson, 2002). Although this approach allows for model averaging so that inferences can be made on the basis of an entire model set, the best model in each analysis in this experiment was well enough supported that I obtained parameter estimates using those models only.

Results

A subset of the experimental replicates showed that the three *Euplotes* clones initially had similar maximum widths of about 73 μm (Figure 3-2, Uninduced treatment. Clone 1: 73.54 μm , S.E. 0.71; Clone 2: 73.55 μm , S.E. 3.88; Clone 3; 73.29 μm , S.E. 1.63). Exposure to predator cues increased this measure of defense in all three clones, but not to the same degree. Clones 1 and 3 had very similar width maxima after induction (Clone 1: 92.42 μm , S.E. 3.95, Clone 3: 91.99 μm , SE 4.14), but that of Clone 2 was larger by nearly 15 μm (107.8 μm , SE 4.69).

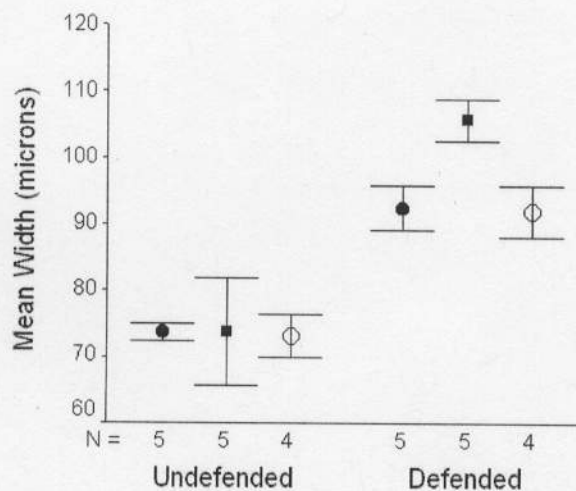


Figure 3-2. Width estimates for defended and undefended *Euplotes*. Measurements were taken 24 hours after exposure to predator cues; data shown are the overall means calculated from 4-5 replicate means, \pm 2 SE (error bars).

E. aediculatus Clone 1 = ● *E. aediculatus* Clone 2 = ■ *E. plumipes* Clone 3 = ○

The best description of the number of *Chlorella* cells consumed as a function of prey density was a Type II functional response in all clones (Table 3-1). There was an effect of inducible defense on the functional response in *E. aediculatus* Clone 2, but not in the other clone of *E. aediculatus* (clone 1) or *E. plumipes* (clone 3). For Clone 2, the model including an effect of defense on the attack rate was supported 1.4 times more than

one in which defense changed the handling time, and twice as much as the Type III model where defense affects the attack rate. A Type II functional response with no effect of defense for *E. aediculatus* Clone 1 was supported two times better than a Type II response with defense affecting either the attack rate or the handling time, and 1.5 times better than the best Type III model. The data were strongly in favour of a Type II functional response with no effect of defense for *E. plumipes* Clone 3. There was no support for a Type I functional response in any of the clones.

When all three clones were analyzed together and the effect of defense excluded, the best Type II model included differences in the attack rate among the three clones (Table 3-2). This model was supported almost 2.5 times more than the case where all three clones had different handling times, and 3 times more than a model where both the attack rate and handling time differed. There was no support for the model where all clones shared an identical functional response. In more detail, a Type II curve fitted to the data from all three clones (Figure 3-3, dashed line) closely approximates the curve fitted for Clone 3 alone (solid line) and Clone 2 when induced, but overestimates the curve for Clone 1 and underestimates that for Clone 2 when not induced. Given that the functional response clearly differs among the three clones, and that the superior model in the combined analysis suggests that these differences are due to the attack rate, I can compare parameter estimates from the individual analyses for each of the three clones. The attack rates for the three clones, for example, ranged from 2.42 in clone 1 to 5.15 in Clone 3 (Table 3-3). In Clone 2, induction of defensive morphology decreased the attack rate from 4.12 to 3.09. Accounting for the sixteen minutes of predation in this experiment, the average maximum predation rate was 5.5 prey/minute/predator (Table 3-3, combined analysis), corresponding to an average handling time of 10.86 seconds/prey ingested.

Table 3-1. Model selection summary: Fit of functional response models for defended and undefended *Euplotes* (average number of *C. vulgaris* algae eaten as a function of prey density). All models were fitted using function nls in program R; 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.

Model (effect of defense)	Log Likelihood	K	AIC	Δ AIC	AIC weight
<i>E. aediculatus</i> Clone 1 (N=99)					
Type I (no effect)	-465.8	3	937.7	6.1	0.012
Type I (slope)	-465.7	4	939.5	7.9	0.005
Type I (intercept)	-465.7	4	939.4	7.8	0.005
Type I (slope and intercept)	-465.6	5	941.1	9.5	0.002
Type II (no effect)	-462.8	3	931.6	0.0	0.262
Type II (attack rate)	-462.6	4	933.1	1.5	0.124
Type II (handling time)	-462.6	4	933.2	1.6	0.118
Type II (attack rate & handling time)	-462.3	5	934.7	3.1	0.056
Type III (no effect)	-463.2	3	932.4	0.8	0.176
Type III (attack rate)	-462.8	4	933.6	2.0	0.096
Type III (handling time)	-462.8	4	933.6	2.0	0.096
Type III (attack rate and handling time)	-462.5	5	935.0	3.4	0.048
<i>E. aediculatus</i> Clone 2 (N=94)					
Type I (no effect)	-478.8	3	963.6	13.5	0.000
Type I (slope)	-475.6	4	959.1	9	0.003
Type I (intercept)	-475.6	4	959.1	9	0.003
Type I (slope and intercept)	-475.3	5	960.5	10.4	0.002
Type II (no effect)	-474.6	3	955.2	5.1	0.024
Type II (attack rate)	-471.1	4	950.1	0	0.308
Type II (handling time)	-471.4	4	950.8	0.7	0.217
Type II (attack rate & handling time)	-471.0	5	952.0	1.9	0.119
Type III (no effect)	-475.3	3	956.5	6.4	0.013
Type III (attack rate)	-471.9	4	951.7	1.6	0.138
Type III (handling time)	-472.0	4	952.0	1.9	0.119
Type III (attack rate and handling time)	-471.8	5	953.6	3.5	0.053
<i>E. plumipes</i> Clone 3 (N=80)					
Type I (no effect)	-393.4	3	792.8	6.6	0.016
Type I (slope)	-393.0	4	794.1	7.9	0.008
Type I (intercept)	-393.0	4	794.1	7.9	0.008
Type I (slope and intercept)	-393.0	5	796.1	9.9	0.003
Type II (no effect)	-390.1	3	786.2	0	0.436
Type II (attack rate)	-389.9	4	787.9	1.7	0.186
Type II (handling time)	-390.0	4	787.9	1.7	0.186
Type II (attack rate & handling time)	-389.9	5	789.9	3.7	0.069
Type III (no effect)	-392.4	3	790.8	4.6	0.044
Type III (attack rate)	-392.3	4	792.6	6.4	0.018
Type III (handling time)	-392.3	4	792.5	6.3	0.019
Type III (attack rate and handling time)	-392.2	5	794.4	8.2	0.007

Table 3-2. Fit of functional response models among clones. Only Type II models were considered, and comparisons were made as to whether shared parameters for attack rate or handling time described the data as well as separate parameters.

Model (effect of clone)	Log Likelihood	K	AIC	Δ AIC	AIC weight
<i>Clones 1, 2 and 3</i>					
Type II (no difference among clones)	-1353.6	3	2713.1	46.9	0.000
Type II (attack rate)	-1328.1	5	2666.2	0	0.561
Type II (handling time)	-1328.9	5	2667.8	1.6	0.252
Type II (attack rate and handling time)	-1327.4	7	2668.4	2.2	0.187

Table 3-3. Parameter estimates: attack rate (c) and handling time (d) from the best Type II functional response models for three *Euplotes* clones. Analyses were conducted for the number of *Chlorella vulgaris* eaten during sixteen minutes of predation, for the three clones separately and with all clones combined. The asymptote (expected maximum predation rate) is given as 1/d.

	Estimate	SE
<i>E. aediculatus</i> Clone 1		
c	2.42015	0.91840
d	0.01845	0.00374
1/d	54.20	
<i>E. aediculatus</i> Clone 2 (uninduced)		
c	4.1235	0.3846
d	0.0070865	0.00228
1/d	141.11	
<i>Clone 2</i> (induced)		
c	3.0928	0.3091
d	0.0070865	0.00228
1/d	141.11	
<i>E. plumipes</i> Clone 3		
c	5.159857	1.463657
d	0.010709	0.001528
1/d	93.38	
<i>All clones combined</i>		
c	3.575182	0.671462
d	0.011314	0.001206
1/d	88.39	

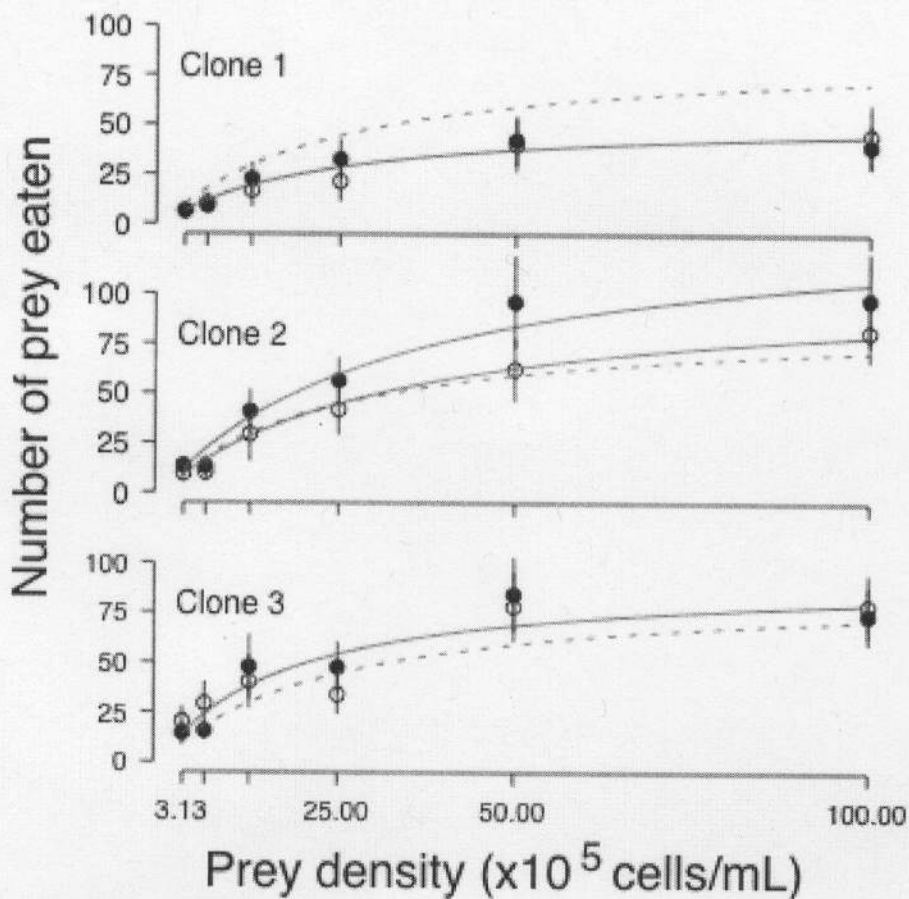


Figure 3-3. Number of prey eaten as a function of prey density by three clones of *Euplotes*. Solid lines are the most parsimonious models within each clone. In clone 2, separate functions are required for defended (open points) versus undefended (solid points) *Euplotes*. The dashed line is the best estimate when clone identity is ignored.

Discussion

This experiment demonstrates clonal variation in the level of defense induction that can be larger than interspecific variation. In addition, the defended forms varied in how much this affected their ability to gather their own resources. This is likely to have implications for the stability of predator prey systems with genetically different prey.

In each case, a model selection approach supported a Holling Type II functional response for the relationship between predation rate and prey density. The induced defense decreased the attack rate in *E. aediculatus* Clone 2, but did not affect the functional response in either of the other clones. These differences in the functional response among clones are associated with a higher level of morphological defense in *E. aediculatus* Clone 2 compared to the other two clones. My 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 inducibly defended species.

My experimental approach had a number of advantages. To begin with, I chose a non-motile algal species as prey to remove any effect of prey motility on the feeding rate of *Euplotes*. Encounter rates with predators could be influenced by motility in prey (Gerritsen and Strickler 1977), as could the predator's capture efficiency or handling time (Dolan and Coats 1991). By taking advantage of the autofluorescence of chlorophyll, I avoided the need to label prey items artificially, which could alter prey cell surface properties in such a way that ingestion rates are decreased (Sanders 1988). Counting the ingested cells microscopically allowed me to detect predation of very low numbers of prey and account for variation among individual predators. The short incubation time of predators with prey eliminated complications due to increases in algal density or depletion of prey by *Euplotes*. Finally, the use of predator cues without live

Stenostomum eliminated predation effects such as loss of *Euplotes* individuals or their prey.

A Type II functional response, in addition to being common in nature, has been observed and modelled in ciliates (Stoeker 1988, Dolan and Coats 1991) and was expected for *Euplotes*. 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 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, Allan 1974). The membrane supply mechanism thus limits ingestion at high prey densities, and is dependent to a certain degree on the rate of digestion. Morphological defense 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 defense) 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, perhaps morphological transformation takes priority under predation and depletes membrane resources at the cost of food vacuole formation. The presence of lateral defense structures may also alter the morphology of the oral region in such a way that fluid currents generated by the AZM for food collection are not as efficient. In either case, a decrease in the feeding rate would indicate that defense influences handling time. However, I did not observe an effect of defense on this parameter of the functional response. This could be due to the experimental design, or it could reflect variation in the level of defense in *Euplotes* populations (Wiackowski et al. 2003, Duquette et al. 2005). A small proportion of defended *E. aediculatus* individuals have maximum widths of up to 120-130 μm , although typically only a few individuals within any population ever reach these extreme sizes at the predator levels I examined. Effective protection from predators must occur at prey widths below these values. The mouth gape sizes of four common

predators of *Euplotes* have been measured at close to or less than 100 μm (Kuhlmann et al. 1999), and predation risk has been estimated to approach zero at about 80 μm width in *Euplotes octocarinatus* (Altwegg et al. unpublished). *Euplotes* cells may be able to maintain a higher maximum rate of ingestion while defended for all but these highest possible levels of defense. If membrane limitation or changed oral morphology as constraints on handling time only become a factor when *Euplotes* cells experience extreme morphological changes (i.e. during periods of unusually high or long-term predation risk or for the largest few individuals within a defended population), any cost of defense on handling time would be minimized.

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 a decreased attack rate in this clone. A predator's attack rate depends in part on its encounter rate with prey, which is in turn determined by the activity levels of predator and prey (Gerritsen and Strickler 1977). In general, when faced with the trade-off between resource acquisition and predation mortality, organisms will alter the frequency and speed of movement so as to maximize fitness (Werner and Anholt 1993). This theoretical prediction has been repeatedly confirmed in diverse taxa, including caddisflies (Kuhara et al. 2001), crayfish (Hazlett and Schoolmaster 1998, Hazlett 1999), waterstriders (Moses and Sih 1998), fish (Johansson and Leonardsson 1998, Lankford et al. 2001, Skalski and Gilliam 2002), and amphibians (Werner and McPeck 1994, Skelly 1995, Peacor and Werner 1997, Relyea and Werner 1999, Anholt et al. 2000, Richardson 2001, Relyea and Yurewicz 2002). As yet, no one has measured the relationship between activity and level of morphological defense in *Euplotes*. Since I did not measure movement speed or frequency in this experiment, I am not certain of their contribution to the different observed functional responses in defended and undefended populations of *E. aediculatus* Clone 2. While defensive morphology itself could alter activity levels, it is also entirely possible that the observed effect of defense on the functional response was the result of reduced foraging. The cytostome in *Euplotes* ciliates is on the ventral cell surface; feeding involves transient attachment to the substrate and generation of fluid currents with the oral ciliature. Because the filtration apparatus and the medium flow

field are perpendicular to the surface, recirculation of water that has already been filtered may pose a problem for the cell (Fenchel 1986). *Euplotes* does however display a compensatory feeding behaviour: walking or swimming short distances at regular intervals between periods of feeding. The frequency of these movements is expected to decrease with increasing predator or resource density (Werner and Anholt 1993). The observation of increased swimming speed in ciliates in response to starvation (Salt 1979, Fenchel and Jonsson 1988, Dolan and Coats 1991) supports these predictions. When resources are in short supply and predators are present, this should result in decreased food intake due to a lower attack rate. *Euplotes* is also known to express evasive behaviour in the presence of *Stenostomum* (Kuhlmann 1994), in which there is an increased frequency of rapid movements away from detected predators. Because *Euplotes* is unlikely to be able to forage during these defensive movements, this should also contribute to a decreased attack rate on its own prey.

Cost-benefit considerations are central to evolutionary theory of inducible defense. The *Euplotes-Stenostomum* system provides an ideal model for asking questions about the relative costs and benefits of induced morphologies and behaviours. As structural features, the development of morphological defenses redirects resources from essential functions like growth and reproduction (Clark and Harvell 1992), whereas defensive behaviours tend 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 defense expression differently, such that morphological defenses are used when food is abundant and behavioural defenses are used when it is not (Van Buskirk 2000). Morphological defense expression increases with resources for a given predator density, but whether behavioural defense is favoured at low food densities has never been examined. It will be interesting to investigate the interplay of morphological and behavioural defenses in future experiments with *Euplotes*. Given enough clones with a weak correlation between behaviour and the level of morphological defense, it should be possible to separate their independent effects.

Ecological studies have revealed the ubiquity of inducible defenses 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 interactions among prey. Less is known about the propagation of trait-mediated indirect effects of induced defenses through food webs (Werner 1992, Werner and Anholt 1996, Peacor and Werner 1997, 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 defenses on these relationships. These data are essential for modelling population dynamic consequences of induced responses. Existing theory argues that inducible defenses are inherently stabilizing (Ives and Dobson 1987). Defenses are more strongly expressed at high densities of predators (e.g. Kuhlmann and Heckmann 1985, Kusch 1993b, Wiackowski and Staronska 1999, Anholt et al. 2000, Duquette et al. 2005), which reduces predation rates with increasing predator density. Defenses are less strongly expressed at high prey densities because of reduced resources (e.g. Wiackowski and Szkarlat 1996, Wiackowski and Staronska 1999, Luttbeg and Schmitz 2000, Turner 2004), which increases predation rates with prey density. These density-dependent feedbacks should be stabilizing, provided that they do not have long time-lags (May 1981, Fryxell and Lundberg 1998, Luttbeg and Schmitz 2000). Variability in prey vulnerability in inducible populations is another property that is expected to contribute to stabilizing the system. Direct tests of these effects have only been done in an alga-rotifer system (Verschoor et al. 2004). This work remains to be done in *Euplotes*.

Chapter 4. General Discussion

I found that variation in the level of inducibility among clones of *Euplotes* was as large as that among species. Because susceptibility to different predators will depend on the size of induced prey, there will also be variation among strains in the effectiveness of the defense. I also found that induction affected the ability of only some strains of *Euplotes* to capture and consume its own prey. This kind of variation can be exploited to test population dynamic models that incorporate inducible defenses. However, to fully understand the consequences of inducible defenses in systems with three or more trophic levels, it is also necessary to quantify the strength of indirect interactions. Since the costs of defense are observed in a reduced growth rate of prey, which is tightly linked to food intake and conversion efficiency, it seems likely the effects of defense are transmitted to other community members through their own functional response.

I restricted my research to defensive morphology, but this is ineffective against predators which are not gape-limited. However, some *Euplotes* species are also capable of expressing induced behavioural responses when exposed to such organisms. For example, *Euplotes* cells move backwards then quickly turn and move in the opposite direction upon contact with the phagotroph *Amoeba proteus* (Kusch 1993a). The frequency of avoidance behaviour in *E. octocarinatus* increases within hours of cultivation with *Amoeba proteus* and allows long-term coexistence of predator and prey (Kusch 1993a). Avoidance of the turbellarian predator *Stenostomum sphagnetorum* has also been observed in *E. octocarinatus* in conjunction with defensive morphology (Kuhlmann 1994). However, behavioural and morphological changes are not necessarily linked; *E. octocarinatus* did not avoid *Amoeba* after defensive morphology had been induced by *Stenostomum* (Kuhlmann et al. 1999). *Euplotes aediculatus*, on the other hand, did avoid *Stenostomum* when preconditioned by *Lembadion bullinum* kairomone and morphologically transformed. It seems then that some avoidance behaviours are predator-specific. Avoidance behaviour of this type is different from the reduced foraging activity in the presence of predators widely reported in metazoans (Lima and Dill 1990). This has not been measured in *Euplotes*, nor have any associated costs.

Inducible adjustments to morphology or behaviour affect interactions within and among species and their population dynamics. Depending on model details, defenses can stabilize or destabilize dynamics. Behavioural responses are almost instantaneous and therefore do not have any destabilizing time lag between perception of predation risk and defense expression. As shown here, morphological changes can also be rapid but are still about one-half generation before complete induction. This may prevent accurate tracking of predation risk (Altwegg et al. 2004). Defenses can be expressed by organisms of any trophic level within a food web, and a stabilizing effect on a pairwise interaction does not necessarily translate to overall community stability. For example, Verschoor et al. (2004) observed a stabilizing effect when resources expressed an inducible defense but the effect was absent when defenses were also expressed by the consumer in a tritrophic food chain. The influence of inducible defenses and other trait-mediated indirect effects are measured in terms of fitness components such as growth and reproduction. The effectiveness of inducible defenses varies among strains and is contingent on the identity and density of predators. This must result in microevolutionary changes in mean phenotype (Inouye and Stinchcombe 2001). The consequences of inducible defenses for stability depend then on the time-scale of coevolution of predators and the other members of food webs that are affected by these defenses.

To conclude, *Euplotes* ciliates, their resources and their predators constitute a model community for investigating inducible morphologies and behaviours. The characterization of the effects of these defenses on fundamental ecological relationships, as I have done here, will facilitate the development and testing of models of community dynamics that realistically incorporate individual variation. Testing the predictions of these models in long-term dynamic experiments with this community will expand our understanding of the factors contributing to community stability.

Future Directions

This thesis outlines what is currently known about the *Euplotes* model system and how it has been exploited to examine ecological questions, but there is still much we do not know. For example, *Euplotes* has a very complex oral apparatus which is designed to direct food towards the cell mouth. This is achieved mainly through the action of the Adoral Zone of Membranelles (AZM), a group of specialized cilia that create fluid currents. Movement of this organelle is visible under the light microscope and indicates active feeding. Measurements of feeding behaviour should account for movement of the AZM as well as cell movement in space. Feeding biology of predators of *Euplotes* is not well known either; information about the size distribution of *Stenostomum* worms and their mouth parts and how these change in response to prey defenses is another open area for research.

I measured the functional response for defended and undefended *Euplotes* at only one density; thus I have not examined interference competition among consumers. Models are available to test for these effects (Skalski and Gilliam 2001), which are likely to be present in natural food webs. Similarly, there is the hidden process of digestion in the handling time parameter of Holling Type II and III models, which theoretically can be separated (Jeschke et al. 2002). Feeding in *Euplotes* is limited in part by digestion in a process that is largely characterized, so it should be possible to separate these aspects of the feeding relationship.

Little is known about the effects of conspecific density on defenses and their consequences in *Euplotes*. While it has been shown that defense level depends on density (Wiackowski and Staronska 1999), this could result from competitive effects (depletion of resources which results in lower defense levels), behavioural effects (the "selfish herd": aggregation to reduce individual predation risk) or both. Gregarious feeding has been observed in *Euplotes*, which supports the selfish herd hypothesis (Lawrence and Snyder 1998). These effects could be experimentally partitioned by exploiting waterborne infochemicals, like the kairomones that induce defense in

Euplotes. We do not know whether *Euplotes* assess conspecific density, but it is likely that they need to do so in order to estimate predation risk and competitive intensity (Peacor 2003). Crude extracts such as freezer-killed conspecifics or purified kairomones could be used to manipulate phenotype and investigate the role of consumer density on defense and to separate changes in behaviour from depletion of resources as a causal mechanism.

Finally, in order to characterize adaptive foraging behaviour in *Euplotes*, we need more tools for manipulating the defensive phenotype. We have not determined the efficacy of freezer-killing any predators other than *Stenostomum*, but other predators of *Euplotes*, like *Amoeba proteus*, could be inactivated this way and may possibly induce behavioural responses without concurrent morphological defense.

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