

Factors affecting the expression of inducible defences in *Euplotes*: genotype, predator density and experience

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Summary

1. Inducible defences alter the strength of interaction in food webs. Their effectiveness depends both on the maximum level of induction and the speed at which induction happens. Maximum level and speed of induction should therefore evolve in concert.
2. We examined the effect of genotype, number of predators and previous exposure to predators on speed and maximum level of induction in a morphological defence expressed by eight clones in three species of the ciliate *Euplotes*.
3. Both speed and maximum level of induction, and the reaction to predator density varied among genotypes. These results show that there is genetic variance for all aspects of this inducible defence and the potential for complex evolutionary change under selection.
4. Higher predator densities led to higher maximum levels of defence and, for one measure of induction speed, more rapid induction. Previous exposure to predators had no detectable effect on either speed or maximum level of induction.
5. Our results demonstrate that *Euplotes* can precisely and rapidly adjust their morphological defence to the magnitude of predation risk. The speed of induction and the maximum level of defence varied among genotypes and this will lead to variation in defence level and vulnerability under natural conditions. Variation in prey vulnerability is a key factor promoting stability in food webs.

Key-words: Phenotypic plasticity, protist, *Stenostomum virginianum*, trait-mediated indirect effects

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Introduction

Inducible defences directly affect food webs by changing the strength of interactions between prey and predators, and between competitors (Anholt & Werner 1999; Werner & Peacor 2003). Exactly what effect they have on community dynamics remains unclear, however. On the one hand, inducible defences have properties that tend to stabilize community dynamics (Ives & Dobson 1987; Vos *et al.* 2004): their expression and effect is density dependent, and inducible defences create variation in prey vulnerability. On the other hand, systems with predators that have saturating functional responses could be destabilized by prey defences (Abrams & Matsuda 1997). Recent theory suggests that not only the maximum level of defence but also the timing of its induction can influence dynamics (Luttbeg & Schmitz 2000), and that longer time lags between exposure to predator cues and adoption of protective morphology

can be maladaptive (Padilla & Adolph 1996; Gabriel 1999). The dynamic consequences of inducible defences depend in part on the ability of prey to respond quickly to predation risk (Altwegg *et al.* 2004). If the inducible defence is reversible, previous encounters with predators may increase the speed and capacity to react in future encounters (for an example see Baldwin & Schmelz 1996). Two crucial aspects of inducible defences 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 defences are expected to evolve when predation risk is variable and unpredictable (Tollrian & Harvell 1999). 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 defences is therefore key for understanding both their evolution and their significance for community dynamics. Interclonal differences in anti-predator 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 defences in plants (e.g. Zangerl & Berenbaum 1990; vanDam & Vrieling 1994;

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English-Loeb, Karban & Walker 1998; Underwood 2000; Underwood *et al.* 2000), animals (Parejko & Dodson 1991; Spitze 1992; Harvell 1998) and protists (Wiackowski *et al.* 2003), none has considered the speed and maximum level of induction simultaneously. Most of these studies examine two levels of induction, defended vs undefended. Few have compared the entire reaction norm among clones (Parejko & Dodson 1991; Underwood 2000).

This study examines variation in the speed and maximum level of induction among species and clones of *Euplotes* ciliates in two separate experiments. In the first experiment, referred to as time course experiment, we exposed *Euplotes* clones to various densities of the predatory turbellarian *Stenostomum* and compared their reaction over time. In the second experiment, referred to as memory experiment, we examined the effect of previous encounters with predators on the speed and maximum level of induction in several clones of *Euplotes*. In response to 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 & Heckmann 1985). The transformed cell dimensions exceed the gape-limit of predators, protecting *Euplotes* from consumption (Kuhlmann & Heckmann 1994).

Measuring the speed of induction is not straightforward if the defence is developed at a non-linear rate, as in our case. We therefore examine two measures for speed of induction: (i) the time needed to reach half of the maximum induction, and (ii) the width reached 6 h after exposure to predators. The first measure is related to the amount of time spent actively transforming, at which stage cell division and therefore population growth rate is strongly decelerated (Kusch & Kuhlmann 1994). This measure may, however, not predict very well how rapidly the defence becomes effective because this depends more on the absolute cell width as the predators are gape limited. Our second measure of speed therefore is such an absolute measure: the cell width reached after 6 h, when all genotypes were actively transforming.

Materials and methods

ORGANISMS AND CULTURING PROCEDURES

We examined inducible defences in several species of *Euplotes* reacting to their turbellarian predator *Stenostomum virginianum* Nuttycombe 1931. *Euplotes octocarinatus* Carter 1972, *E. aediculatus* Pierson 1943 and *E. plumipes* Stokes 1884 were kindly supplied by K. Wiackowski at Jagiellonian University, Cracow, Poland (see also Wiackowski *et al.* 2003). All *Euplotes* isolates originated from single cells and were since kept as clonal populations in a liquid medium consisting of 0.04% crushed protozoan pellets (No. 13–2360, Carolina Biological Supply Company, NC) in mineral water (NAYA, Mirabel, Québec, Canada) filtered through double-layered no. 4 coffee filters (Thrifty Foods, Inc.,

Canada). Inoculates of *Bacillus cereus* (Boreal Laboratories, St. Catherines, ON, Canada) 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. We used either live or freezer-killed *Stenostomum* worms (~350 worms ml⁻¹, stored in 1.5-ml Eppendorf tubes, at -4 °C) to induce prey defences in all experiments.

TIME COURSE OF DEFENCE

The objectives of this experiment were (i) to characterize the time course of morphological transformation in *Euplotes* at several predator densities and (ii) to establish interspecies and interclonal variation in the time course of defence. To examine these objectives, we 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 24–36 h.

On each of three different dates, we 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 we therefore ended up with two to six replicates in total for each treatment. Live *Stenostomum* predators (2, 4, 8 or 16) were counted from dense cultures into wells in 500 µl culture fluid. The addition of live predators did not lead to significant size-selective predation, as the observed size changes of the ciliates exceeded the variation in their initial population by far. We added 500 µl NAYA water to zero-predator control wells. *Euplotes* cells were transferred from well-established cultures and pooled in fresh bacteria-inoculated medium, then 150 cells were counted into experimental wells in 500 µl medium. The time of addition of each *Euplotes* clone was time zero for that set of replicates.

The time course of defence was examined at different predator levels by photographing each well at 2, 6, 12, 18, 24 and 36 h postinduction. We scanned the flat bottom of each well systematically with an inverted microscope (Leica DM-IRB Wetzlar, Germany) at 100× magnification and captured images through a Cohu CCD camera (San Diego, USA) of the first 10 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 for 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. We 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 6 h, and the time until half of the maximum cell width was reached. The variation in defence 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 & Gentleman 1996).

MEMORY IN DEFENCE

The objectives of our second experiment were to (i) determine whether past exposures to predators has any effect on the speed and maximum level of induction of the defended form in *Euplotes* (memory effect), and (ii) to establish potential variation in memory between species and clones. We investigated these objectives using a similar experimental design as Baldwin & 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 treatments were 001, 011, 101 and 111. Between episodes, the predation cues were removed for 2–3 weeks and the cells regained their undefended form. During the final episode, all experimental units received the predation cue, and we compared speed and amount of induction of prey that had been exposed to predators 0, 1 and 2 times before the final induction. The predation cue consisted of frozen *Stenostomum* worms (~350 ml⁻¹). The experimental units were individual wells in 24-well culture plates (Costar, Corning, USA) 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. We arbitrarily chose two clones of *E. aediculatus*, two clones of *E. octocarinatus* and one clone of *E. plumipes* for this experiment, and replicated the whole experiment six times in total, distributed over two temporal blocks. Treatment 001, where *Euplotes* were induced only at the end, was doubly replicated to increase the power of the comparison for naive cells vs the treatments with prior 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 h after the final exposure to predator cues, we 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 facilitate data collection. Preservation may affect the cell shape of ciliates (Chaput & Carrias 2002).

Taking the measurements immediately after the preservation, we did not see any shape changes but avoid comparing these measurements with the ones of live cells. Measurements of live cells were also taken before and 48 h after adding the predator cue during the first two episodes to verify that our treatments responded as they should. We used cell width 6 h and 36 h after the final induction as a measure of speed and maximum level of induction. These data were analysed using mixed effects models in procedure lme of program R 1.8.1 (Ihaka & 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 DEFENCE

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

Euplotes responded to predators with a dose-dependent increase in width that was measurable within 6 h and completed in 12–18 h (Fig. 1). *E. 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 2-h postinduction. Separation between the zero predator control and predator treatments in the other species and clones was not as obvious until about 6 h postinduction. Widths in the zero predator controls remained near baseline values for all clones, with the exception of *E. octocarinatus* 4, which increased slightly over the course of the experiment, and *E. aediculatus* 3, which decreased.

We measured the speed of induction in two different ways: (i) as the time needed to reach half maximum induction and (ii) as width 6 h 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 we found no overall difference among species in their speed of reaction to predators (Table 1). However, clones within species did differ in

Table 1. Time course experiment. ANOVA summary showing the effects of predator number, species, and clone (nested within species) on speed of induction, measured as time to half maximum induction. This analysis only included treatments with predators present

	df	Sum of squares	Mean square	F	P
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		

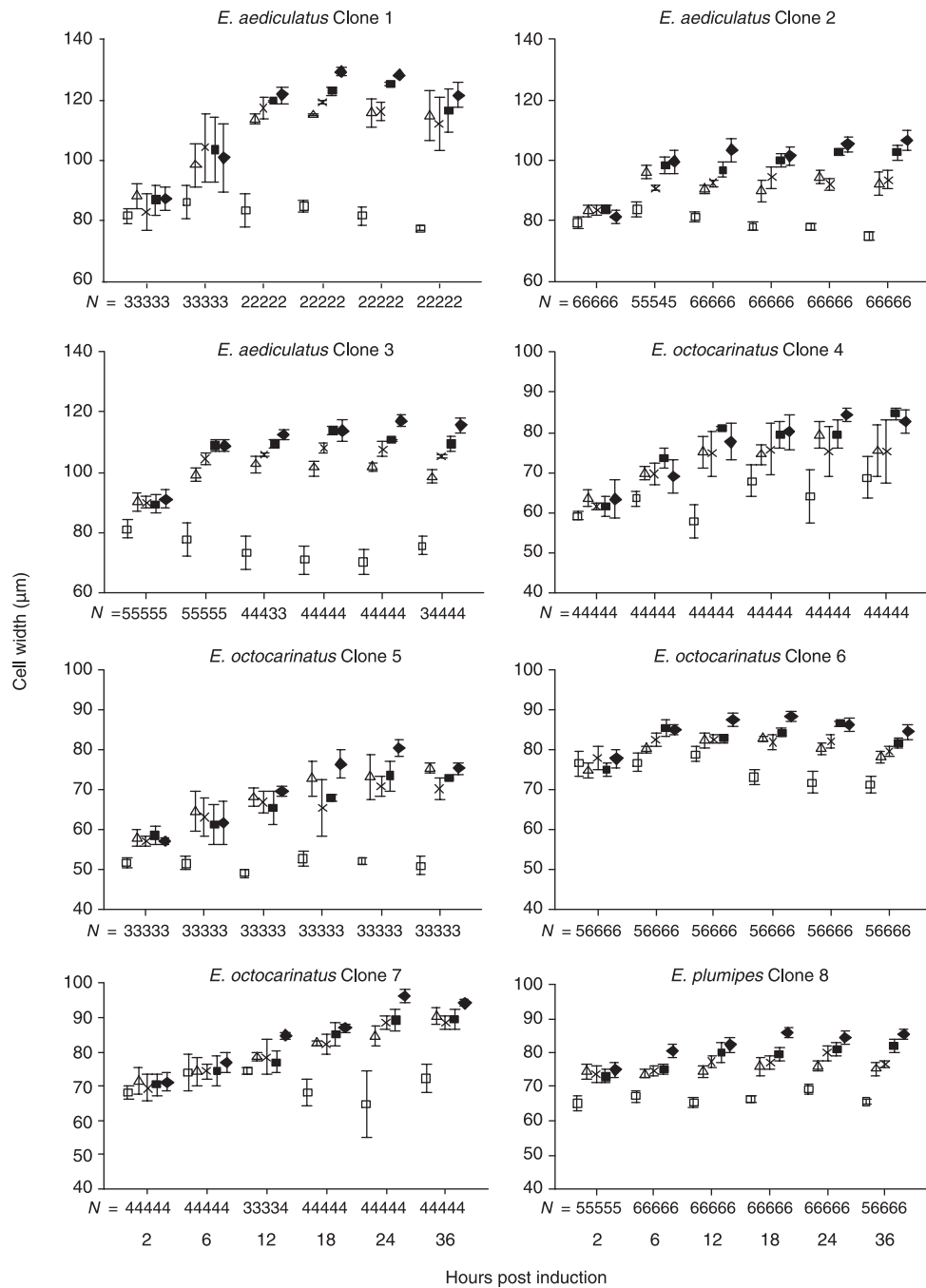


Fig. 1. Time course experiment. Time course of a defensive morphological reaction norm in eight genotypes of *Euplotes*. The treatments are: \square 0, \triangle , \times , \blacksquare , \blacklozenge 16 predator individuals per unit. Data points represent the means (\pm standard error) of two to six experimental replicates per treatment (N), using the median width of 10 cells per replicate.

the speed of induction, variation which appears to be primarily among clones of *E. octocarinatus* (Fig. 2a). Clones 4 and 6 responded particularly rapidly, reaching half maximum induction within 6 h, almost two times more quickly than clone 5 at 10 h and three times more quickly than clone 7 at 16 h. (ii) At 6 h, cells were on average 17.67 μm wider when exposed to predators than in the predator-free treatment (Table 2, Predator presence), and their width increased with predator density by 0.23 μm per predator (Table 2, Number of predators). We found significant differences among species and clones in the width at 6 h (Fig. 2b, Table 2).

As clones and species differed in their undefended cell width, the information 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), and this result is thus identical to the one for time to half maximum induction (i, above).

We found significant differences among species and clones in the maximum width obtained upon exposure to predators (Table 3, Fig. 3). Again, the interaction terms between predator treatment and clone/species

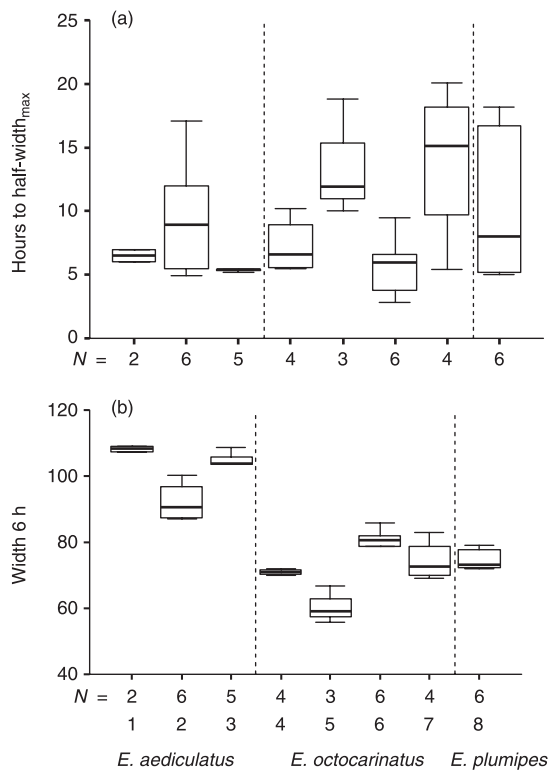


Fig. 2. Time course experiment. Interspecies and interclonal variation in the speed of induction in *Euplotes* exposed to eight predatory *Stenostomum* worms. Induction speed was measured as the time taken to reach half maximum induction (a), and the cell width 6 h after exposure to predators (b). Data show the 50th percentiles for median values from two to six experimental replicates, and extend from the maximum to the minimum estimates.

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 3). *E. octocarinatus* clone 5 did not react as strongly as the other clones within that species (Fig. 3). Clones 4 and

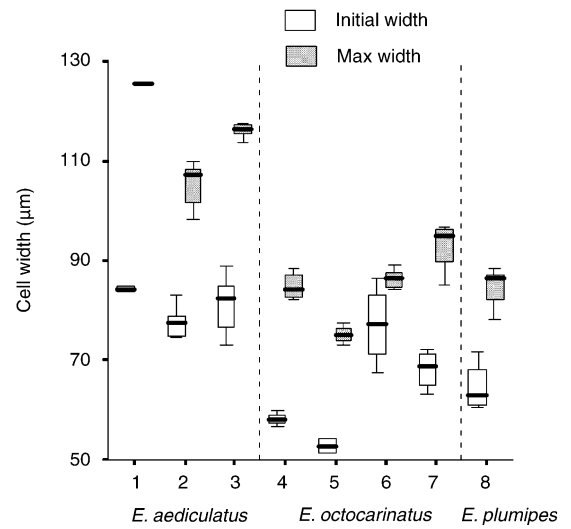


Fig. 3. Time course experiment. Interspecies and interclonal variation in maximum induction in *Euplotes* after exposure to eight predatory *Stenostomum* worms. Initial width is the cell width expressed in the predator-free control treatments.

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* (Fig. 3).

MEMORY IN DEFENCE

Here, we exposed two clones of *E. aediculatus*, two clones of *E. octocarinatus* and one clone of *E. plumipes* to predator cues one to three times, and asked whether previous exposure leads to more rapid induction or a higher maximum level of induction at the final episode. We found no effect of previous induction on cell width 6 h (our measure of speed of induction) or 36 h (maximum level of induction) after the final exposure to the predator cue (Fig. 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 we treated the number of previous exposures as a covariate, or contrasted the treatment with no previous exposure to all other ones (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 6 h: -0.96 , SE = 3.02). There was also no significant difference among genotypes in a potential memory effect regardless of how we analysed the number of exposures (interaction between treatment and genotype: all $P > 0.07$).

Discussion

In two experiments, we examined the effects of predator density and previous exposure to predators on speed and maximum level of induction of a morphological

Table 2. Time course experiment. ANOVA summary table showing the effects on cell width 6 h 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	2 802.70	2802.70	154.24	< 0.001
Number of predators (NP)	1	208.60	208.60	11.48	0.001
Species	2	1 988.30	994.20	0.82	0.493
Block	2	18 258.80	9129.40		
PP × Species	2	804.20	402.10	3.16	0.130
NP × Species	2	85.10	42.60	3.80	0.099
Clone (Species)	5	6 086.70	1217.30	67.00	< 0.001
PP × Clone (Species)	5	636.00	127.20	7.00	< 0.001
NP × Clone (Species)	5	56.00	11.20	0.62	0.688
Residuals	152	2 761.90	18.20		

Table 3. Time course experiment. ANOVA summary table showing the effect of predator presence (PP), number of predators (NP), species and clones (nested within species) on maximum cell width. See legend of Table 2 for more details

	df	Sum of squares	Mean square	F	P
Predator presence (PP)	1	7 998.30	7998.30	373.67	< 0.001
Number of predators (NP)	1	1 592.60	1592.60	74.41	< 0.001
Species	2	17 479.20	8739.60	10.27	< 0.02
Block	2	3 125.00	1562.50		
PP × Species	2	1 031.30	515.70	2.95	0.14
NP × Species	2	240.80	120.40	18.52	< 0.01
Clone (Species)	5	4 254.40	850.90	39.75	< 0.001
PP × Clone (Species)	5	873.50	174.70	8.16	< 0.001
NP × Clone (Species)	5	32.60	6.50	0.30	0.91
Residuals	152	3 253.50	21.40		

defence in eight genotypes of the ciliate *Euplotes*. While *Euplotes* precisely adjusted their level of defence to predator density, there was no detectable effect of previous predator exposure on either speed or maximum level of induction. The genotypes strongly differed in their speed of reaction, in the maximum level of defence and in their reaction to predator density. The variation between clones was often larger than the variation between species.

Our 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 & Heckmann 1985; Kusch 1993, 1995; Wiackowski & Szkarlat 1996; Kuhlmann, Kusch & Heckmann 1999) and other organisms (Anholt, Werner & Skelly 2000; Underwood 2000; Van Buskirk & Arioli 2002; Relyea 2004) showing that the level of defence increases with predator or herbivore density. Most genotypes in our experiment reacted strongly to the lowest predator density and then increased their defence only by smaller amounts when exposed to higher predator densities (see Fig. 1). This suggests that the ecological effects of this inducible defence are strongest when predator densities vary around

very low densities, because at high predator densities all prey tend to be well protected. On the other hand, the defence may be effective only when expressed at a high level and further small increases in defence level may translate into large differences in vulnerability. Kusch (1995) examined the relationship between defence level in *Euplotes* and predation risk by *Stenotomum* and found that this morphological defence becomes effective at about 85 µm cell width, which *E. octocarinatus* and *E. plumipes* only reached at high predator densities in our experiment. In contrast, we have earlier found that already low levels of defence are very effective and higher levels of defence do not further decrease vulnerability much (R. Altwegg *et al.*, unpublished observations). It depends in part on the size of these turbellarian predators.

We found variation among genotypes for all aspects of this morphological defence: speed of induction, maximum level of induction, and the relationship between predator density and defence 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 rapid reaction can ensure that the phenotype matches the environment (Padilla & Adolph 1996; Gabriel 1999). West-Eberhard (1989) proposed that the lability of a trait may influence the evolution of plasticity in that trait. Yet, speed of induction for a defence has rarely been examined (but see Van Buskirk 2002), and we know of no study that has examined genetic variation in this trait. Variation for the maximum level of induction, on the other hand, has previously been found in *Euplotes* (Wiackowski *et al.* 2003), some animals (Parejko & Dodson 1991; Spitz 1992; Harvell 1998) and plants (Zangerl & Berenbaum 1990; vanDam & Vrieling 1994; English-Loeb *et al.* 1998; Underwood *et al.* 2000). We found that the variation between clones was generally larger than the variation between species. This highlights the need to consider several genotypes when comparing inducible defences among species.

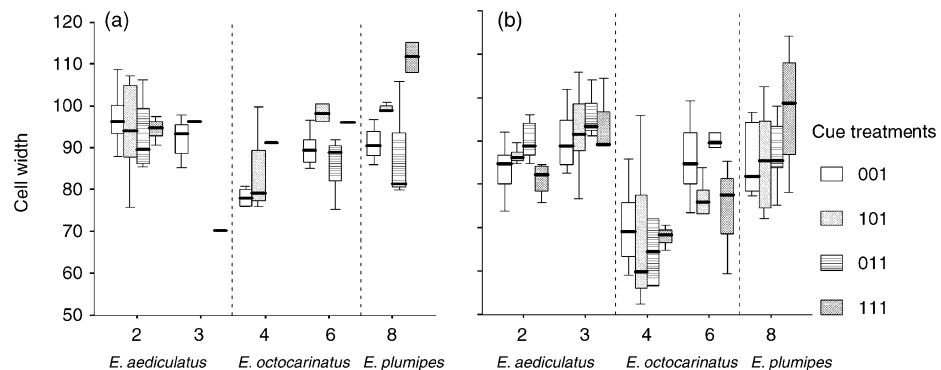


Fig. 4. Memory experiment. Effect of prior induction on morphological antipredator defence in *Euplotes* (a) 6 h and (b) 36 h after exposure to predator cues. During three episodes, *Euplotes* were exposed to a predator cue (1) or control medium (0). The figure shows measurements taken during the final episode when all treatments were exposed to the predator cue. At 6 h, some replicates of *E. aediculatus* clone 3 and *E. octocarinatus* clone 4 had too low ciliate densities to yield measurements. We obtained measurements from all replicates at 36 h, though, after fixing the ciliates with Lugol's solution.

Our second experiment revealed no indication that previous predator exposure affects the speed or maximum level of induction in *Euplotes*. The most familiar case of such a memory effect is the vertebrate immune system, which is an inducible defence against pathogens. Furthermore, sessile marine invertebrates can show stronger aggressive responses against competitors they are familiar with (review in Harvell 1990). Other than this, memory effects in inducible antipredator defences have apparently been investigated only in one case: the tobacco plant *Nicotiana glauca* produces nicotine upon attack by herbivores and does so more rapidly if it was previously attacked (Baldwin & Schmelz 1996). *Euplotes* may already maximize the speed of induction, and in contrast to chemical defence or immune systems, it may not be possible to store components of this morphological defence over long periods of time. In our experiment, 2–3 weeks elapsed between inductions, and this corresponds to 10–20 cell cycles, which may have been too long to find a memory effect. On the other hand, this defence is maintained during cell division and we wanted to be sure that *Euplotes* had completely lost their previous induction. If a partially induced *Euplotes* cell re-encounters a predator, we expect it to regain full induction quickly just because part of the defence is already expressed. In an earlier experiment, we found that *Euplotes* continually adjust their defence and this led to changes in the defence level on a similar time-scale as the population dynamics of prey and predators (Altwegg *et al.* 2004).

In conclusion, our experiments showed that predator density and genotype affect the expression of inducible defences in *Euplotes*. This is important for understanding both the evolution of inducible defences and their effect on community dynamics. On the one hand, our results demonstrate that *Euplotes* can precisely and quickly adjust their defence to the current predation risk and that there is scope for complex evolutionary change in this trait. On the other hand, our results 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 & Lenski 1999; Vos *et al.* 2004).

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