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Research article

Testing for resistance of pelagic marine copepods to a toxic dinoflagellate

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Abstract. With few exceptions, the evolutionary consequences of harmful algae to grazers in aquatic systems remain unexplored. To examine both the ecological and evolutionary consequences of harmful algae on marine zooplankton, we used a two-fold approach. In the first approach, we examined the life history responses of two geographically separate *Acartia hudsonica* (Copepoda: Calanoida) populations reared on diets containing the toxic dinoflagellate *Alexandrium fundyense*. One copepod population was from a region, Casco Bay, Maine, USA, that has experienced recurrent blooms of highly toxic *Alexandrium* spp. for decades; whereas the other population from Great Bay, New Jersey, USA, has never been exposed to toxic *Alexandrium* blooms. The life history experiment demonstrated that when the copepod population from New Jersey was reared on a diet containing toxic *A. fundyense* it exhibited lower somatic growth, size at maturity, egg production and survival than the same population reared on a diet without toxic *A. fundyense*. In contrast, toxic *A. fundyense* did not affect the life-history traits of the Maine population. Fitness, finite population growth rate (λ), was significantly reduced in the New Jersey population, but not in the Maine population. These results are consistent with the hypothesis of local adaptation (resistance) of the historically exposed copepod population to the toxic dinoflagellate. In the second approach, we further tested the resistance hypothesis with a laboratory genetic selection experiment with the naïve New Jersey copepod population exposed to a diet containing toxic *A. fundyense*. This experiment demonstrated that the ingestion and egg production of adult females of naïve copepods fed *A. fundyense* improved after three generations of being reared on a diet containing the toxic dinoflagellate. The results of the present study have important implications for understanding how grazer populations may respond to the introduction of toxic algae to their environment, and suggest that grazer resistance may be a feedback mechanism that may lead to bloom control.

Key words: *Acartia hudsonica*, *Alexandrium fundyense*, toxic algae, life history, biogeography, rapid evolution, life table

Introduction

Harmful algal blooms (HABs) are occurring in previously unaffected ecosystems and their effects on aquatic ecosystem processes are not fully understood (Hallegraeff, 1993). Recently, it has been shown that the ecological relationship

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between zooplankton grazers and toxic algae is closely shaped by their evolutionary history (Hairston *et al*., 1999, 2002; Colin and Dam, 2002a). Freshwater studies have shown that populations of *Daphnia* sp. from lakes where toxic cyanobacteria have bloomed for generations have evolved resistance to the toxic algae (Gilbert, 1990; Hairston *et al*., 1999; Hairston *et al*., 2002). Resistance has enabled the *Daphnia* to feed and grow at higher rates in the presence of the toxic cyanobacteria than conspecifics never exposed to the toxic algae. Colin and Dam (2002a) demonstrated that the ability of adult females of the marine copepod *Acartia hudsonica* to feed and produce eggs on a diet containing the toxic dinoflagellate *Alexandrium fundyense* was related to whether toxic *A. fundyense* blooms occur in the regions from which the copepods originated (here termed exposure history). Colin and Dam (2002a) suggested that these differences were due to evolved resistance in the copepod populations exposed to blooms of toxic *Alexandrium*.

Traditional frameworks used to examine the grazer–toxic alga relationship, in which effects of the toxic alga are examined only on adult zooplankton (Teegarden and Cembella, 1996; Turner *et al*., 1998, Teegarden, 1999, Frangópulos *et al*., 2000; Colin and Dam, 2002a, b; Liu and Wang, 2002) have yielded disparate results partly because of uncertainty as to whether the individuals employed in the experiments came from resistant populations. Furthermore, such studies on adult stages alone are not sufficient to test for grazer resistance against toxic phytoplankton because population fitness was not measured. One approach to test the resistance hypothesis is to use life history tables, in the context of population exposure history, to examine how toxic algae affect the demographic traits (e.g. survival, age at maturation, fecundity, time to reproduction) and fitness (finite population growth rate) of zooplankton populations. First, comparative demographic studies are essential to test for natural selection. In addition, this approach examines the effect on all life stages, thereby allowing us to identify which stages and demographic traits are most affected by the presence of toxic algae. By comparing the life history effects on resistant versus non-resistant zooplankton populations, we can learn which traits have evolved in the resistant population; and from the non-resistant population, we can learn what demographic traits are negatively affected by toxic algae and reduce grazer population growth.

In this study, we expand upon the original work of Colin and Dam (2002a), and use a two-fold approach to test whether marine copepod populations historically exposed to toxic dinoflagellate blooms have evolved resistance. In the first approach, we used a life table analysis to determine how the exposure history of copepod populations to toxic *A. fundyense* is related to their life history traits and fitness when fed diets containing toxic *A. fundyense*. Specifically, we followed cohorts of naïve (from Great Bay, New Jersey) and historically exposed (from Casco Bay, Maine) *Acartia hudsonica* reared

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throughout their life cycle on diets with and without toxic *A. fundyense*. We compared somatic growth, size at maturity, time to reproduction, survival and egg production, and the finite rate of natural increase, λ, in both cohorts. In the second approach, we performed a laboratory genetic selection experiment to examine if rearing the naïve New Jersey *Acartia hudsonica* population on a diet containing toxic *A. fundyense* may change the fitness traits of individuals in the population when fed toxic *A. fundyense*.

Materials and methods

Collection and culture of organisms

Populations of *Acartia hudsonica* were collected from Casco Bay, Maine (ME; 43°39′N, 74°47′W), and Great Bay, southern New Jersey (NJ; 39°23′N, 74°47′W). Casco Bay experiences recurrent blooms of toxic *A. fundyense*, whereas Great Bay has never experienced an *A. fundyense* bloom (Cohn *et al.*, 1988; Anderson *et al.*, 1994). Copepods were collected with a 200 μm mesh net and transported to the laboratory within 24 h of collection.

The copepods used in the life history and genetic selection experiments came from laboratory cultures of copepods collected from the different sites. High densities were maintained in the cultures (500–1000 individuals) to avoid inbreeding (Colin and Dam, 2002a). Both copepod populations were reared in these cultures under identical conditions (12–15 °C and 12 h : 12 h light–dark regime) for over 11 generations (Colin and Dam, 2002a). Animals in the cultures were fed, what we term, their 'standard diet' ($\sim$ 500 μg CL^{-1}) consisting of a mixture of equal proportions of *Thalassiosira weissflogii*, *Isochrysis galbana* and *Rhodomonas lens* (Feinberg and Dam, 1998). Rearing all of the copepod populations at the same temperature, light and food regimes for several generations eliminated both maternal effects and environmental variance. This allowed us to attribute the observed differences among populations to genetic variance (Falconer, 1996, pp. 122–144).

For the life history experiments, copepods were fed either the 'standard diet' (control) or a toxic diet (treatment). Toxic *A. fundyense* (strain NB-05, toxin content = 12.4 $\pm$ 4.1 pg STXeq. per cell), was added to the 'standard diet' to make the toxic treatment diet (= 75% standard diet + 25% toxic *A. fundyense* by carbon). All algal cultures were grown in F/2 medium (Guillard, 1975) at 14 °C with 12 h : 12 h L/D cycle. Cultures were maintained in exponential growth by replacing half of the cultured medium with fresh medium each week. The concentration of *A. fundyense* in the experiments was set to be within the range reported during natural *Alexandrium* sp. blooms. Copepods were kept under the same temperature and light conditions as during rearing.

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To measure toxin content of *A. fundyense,* toxins were extracted from replicate aliquots according to Anderson *et al.* (1994) and analyzed by HPLC using methods of Oshima *et al.* (1989) in our laboratory (the source for the saxitoxin (STX) standards was NRC, Halifax, Canada). Of the suite of saxitoxins present in *A. fundyense,* we quantified the most potent – STX, neosaxitoxin (NEO) and gonyautoxins I–IV (GTX 1–4) (Schantz, 1986; Indrasena and Gill, 1999). This was sufficient to confirm the toxic nature of *A. fundyense*.

Measurement of life-history traits

We compared the life-history traits of copepods from both the ME and NJ populations reared on the control diet (named the NJ- and ME-control cohorts) or the treatment diet (named the NJ- and ME-treatment cohorts) by examining three replicate cohorts (initially 60 copepods per cohort) per diet for each population. These cohorts were examined throughout the life span of the copepods to measure life-history parameters. Two experiments were performed, one in which the individuals in the cohorts were reared from naupliar through adult stages (referred to as the whole life experiment) and another in which adults were reared from C-V stage (last copepodite stage) to death (referred to as the adult survival experiment). The methods were the same for each experiment.

The experimental set-up was designed to provide the cohorts with a relatively constant and high food concentration over the duration of the experiment. Accordingly, the cohorts were raised in 1 L polycarbonate cylinders with a 30 and 200 μm mesh bottoms, during juvenile and adult stages, respectively, that were placed into a 20 L bucket containing the control or a bucket containing the treatment diet (totaling three ME and three NJ cohorts per bucket). With this design a bucket effect is possible, however, as our results show, the interaction between population and treatment demonstrate that there was not a bucket effect. The cylinders were gently lifted and lowered daily to mix their contents, and the contents of the bucket were lightly bubbled to maintain an aerated and mixed food medium. Food concentrations were maintained at 250 μg CL^{-1} for the naupliar stages and 600 μg CL^{-1} for the copepodite and adult stages. At a total food concentration of 600 μg CL^{-1}, the concentration of the standard diet within the treatment diet exceeds the feeding saturation level of *Acartia hudsonica* females (Colin and Dam, unpublished data). Hence, differences in life-history traits between the control and treatment copepod cohorts cannot be ascribed to differential food limitation related to the standard diet.

To start the cohorts, approximately 600 eggs produced by more than 500 adults were randomly removed from the ME and NJ cultures, incubated in a solution containing the standard diet, and kept at 15 °C for 3 days. Upon hatching, 60, nauplii were placed into each of the 1 L polycarbonate cylinders.

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Every 2 or 3 days, the cylinders were gently lifted out of the buckets and the copepods were gently rinsed from the cylinder meshes into petri dishes filled with filtered seawater. Copepods were examined under a dissecting microscope, survivors recorded and dead individuals removed. The copepods were also video recorded with a Pulnix® camera attached to the dissecting microscope for later analysis of body size (see below). Then, the copepods were immediately returned to the cylinders. When individuals reached adulthood, egg production rate was recorded on two consecutive days. The food solutions in the buckets were replaced each time the copepods were examined. Food concentration fluctuated $<25\%$ throughout the duration of the experiment.

The total length (for nauplii) or prosome length (for copepodites and adults) of 20 copepods from each cohort was measured from the video using the Optimas® image analysis software.

The whole life experiment was terminated before all of the adult copepods died. Therefore, adult survival was analyzed in a separate experiment, which followed the same procedure detailed above. To start the experiment, 150 copepodites in the C-IV stages were removed from the NJ and ME populations and incubated at 15 °C for 2 days in beakers. Individuals that had molted into adult copepods (15 males and 15 females) were then picked from the beakers and placed into the 1 L polycarbonate cylinders (again, three control and three treatments per population). As in the whole life experiment, they were removed from the cylinders and checked every 2–3 days until no individuals were left. The number of survivors was counted, but copepod lengths were not measured in this experiment. As for the other experiment, egg production rate was recorded on 2 days.

Analysis of life-history data

We estimated survivorship, life-stage duration, age at maturity, size at maturity, somatic growth and fecundity to compare the life-history effects of toxic *A. fundyense* on the naïve and historically exposed copepod populations. Survivorship, l_x, the probability of surviving to age x, was calculated as:

$$l_x = n_x/n_0 \tag{1}$$

where n_x and n_0 represent the number of individuals alive at age x and at age 0, respectively. In order to identify the age with the greatest risk of dying, we calculated the hazard function, h_x (Lee, 1980):

$$h_x = f_x/l_x \tag{2}$$

where f_x is the probability density function:

$$f_x = (n_x - n_{x+1})/(n_0((x+1) - x)). \tag{3}$$

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Survivorship for censored (i.e. experiment was terminated before death of last individual) and uncensored data (experiment continued until the death of the last individual) was compared using the Gehan–Wilcoxon non-parametric test (Lee, 1980; Pyke and Thompson, 1986). We employed the Statview® version 5.0.1 software for all statistical analyses.

To determine the age to maturity and life-stage duration, we used the median development time (Peterson, 1986; Carlotti and Nival, 1991), which is defined as the age, x, at which 50% of the individuals reached a specific stage (e.g. maturity). We calculated the median development time from the regression of the percent copepodites or adults in the cohort versus days (Peterson, 1986). The size at maturity and somatic growth were determined using prosome length measurements (after about the C-IV stage copepod sex could be determined and only female sizes were measured).

Age-specific fecundity, m_x, was defined as the number of eggs per female per day. Since it has been shown that female egg production in *Acartia hudsonica*, feeding on diets with and without toxic *A. fundyense*, is a function of food concentration (Colin, 2002), and food concentration was held relatively constant, m_x was determined on only two dates for each experiment. The mean m_x was then used for fitness estimates.

We estimated the fitness (λ = finite population growth rate) of the individual cohorts using two different population models, age-classification and stage-classification. The age-classification model employed measurements of l_x and m_x in population projection matrices (Leslie matrices) using a projection interval of 3 days. We calculated the survival probabilities, P_x, as:

$$P_x = l_{x+1}/l_x \tag{4}$$

and fertilities, F_x, assuming a 1 male : 1 female ratio as:

$$F_x = P_0\, m_x/2 \tag{5}$$

using the birth-pulse model (Caswell, 1989; Ebert, 1999). Finite growth, λ, was calculated as the dominant eigenvalue of each matrix.

The stage-classification model (Ebert, 1999) used eggs, nauplii, copepodites and adult stages (Fig. 1). Since previous work has shown egg hatching to be unaffected by toxic *A. fundyense* (Colin and Dam, 2002a), egg survival was assumed to be 1. For this model, we calculated the probability of progressing to the next stage, g_x, as the (fraction leaving) × (survival per day) and the probability of staying in the same class, s_x, as the (fraction staying) × (survival per day). Survival per day is calculated as:

$$\text{Survival per day} = (l_b/l_e)^{1/d} \tag{6}$$

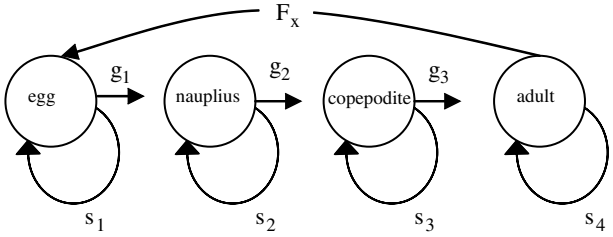

Figure 1. Life cycle for the stage class model used to estimate fitness. *F* is fecundity, *g* is probability of transferring to the next stage and *s* the probability of staying in the same class. See the 'Materials and methods' section for explanation of *g* and *s*.

where l_b and l_e are the survival at the beginning and end of the stage, respectively, and d is the stage duration. Fraction leaving a stage is $1/d$ and fraction staying is $1-(1/d)$ (Ebert, 1999).

The population growth rate, λ, was calculated from the stage-classification model using the original censored adult stage data from the whole life experiment and by inserting the complete adult survival data from the adult survival experiment. Again, λ was calculated as the dominant eigenvalue of each matrix.

Within each model used, λ values for the different populations and treatments were compared using the non-parametric Mann–Whitney *U*-test treating the triplicate cohorts of each treatment as replicates (Sokal and Rohlf, 1995).

Genetic selection experiment

We exposed the naïve *Acartia hudsonica* population from Great Bay, New Jersey, USA, to a diet containing toxic *A. fundyense* and examined the effects of *A. fundyense* on the copepods for five generations.

Before we reared any copepods on diets containing toxic *A. fundyense,* we measured the ingestion and fecundity of adult females of the naïve New Jersey copepods from duplicate cultures (i.e. generation = 0; Fig. 2). At the same time we randomly collected eggs from each of the two cultures and split them into two separate lines, each consisting of two cohorts, with 300 eggs in each cohort. The cohorts in the control line were reared on the 'standard diet' whereas the cohorts in the *Alexandrium* line were reared on a diet consisting of 75% 'standard diet' + 25% toxic *A. fundyense* by carbon. This step in the experiment (unnumbered generation after generation 0 in Fig. 2) effectively started the process of genetic selection for grazer resistance to the toxic dinoflagellate. Both diets were provided at concentrations (about 600 μg CL^{-1}) typically in excess of the saturation point of the copepod's ingestion and egg production (Colin and Dam, unpublished data). Hence, animals were not food-limited.

Because the phenotypic response of each line would be a function of both its genetic pool and the diet to which it was exposed, to ascertain differences

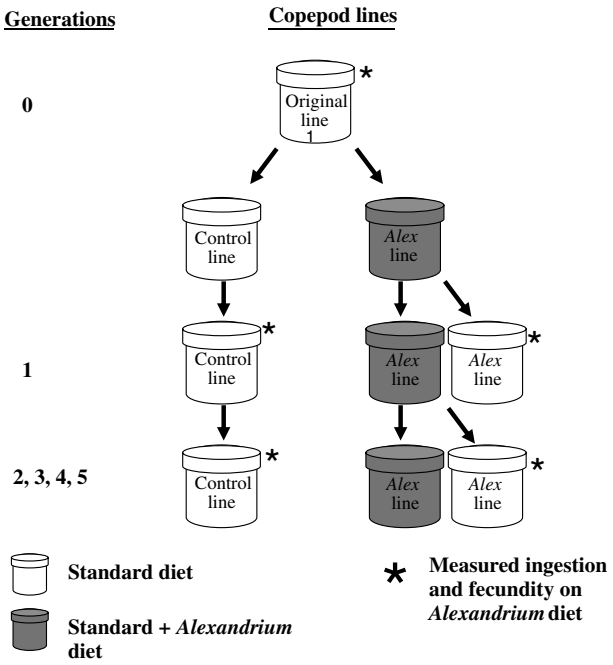

Figure 2. Schematic of experimental design of laboratory genetic selection experiment.

between the two lines we used adult females from both lines that were reared on the standard diet (Fig. 2). For this purpose, we initially reared half of the offspring produced from the *Alexandrium* lines in a separate container on only the standard diet (same as the control line) and only copepods from these separate containers were used to measure adult female ingestion and egg production rate (generation 1 in Fig. 2). This procedure was then repeated for all subsequent (2–5) generations. In essence, this procedure allowed for continuous selection during the entire experiment for grazer resistance in the *Alexandrium* line while allowing comparison of animals from both lines reared on the standard diet. At least 300 eggs (about half of an entire clutch) were used to start each cohort throughout the experiment, except that to start generation 1 in the *Alexandrium* lines we only used about 100 eggs. This was the result of an almost immediate population bottleneck that occurred during generation 0 in the *Alexandrium* lines.

To compare the effects of toxic *A. fundyense* between lines on each generation, we measured the ingestion and fecundity of adult female *Acartia hudsonica* from each of the cohorts (two control and two offspring of *Alexandrium* lines) fed 150 μg CL^{-1} of toxic *A. fundyense*. Before measuring ingestion, copepods were acclimated on the diet at experimental conditions (same as rearing conditions) for 48 h. Then, triplicate sets of 12 individuals from each cohort

were placed into 600-mL bottles containing the diet solution. Two bottles without copepods served as controls. Bottles were incubated for 24 h and rotated end over end at 1.3 rpm. At the end of the incubation, eggs and algal samples were collected and counted. Initial and final algal concentrations were measured using the Utermöhl (1958) technique. Ingestion rates were calculated from cell disappearance using equations from Frost (1972). Egg production rate was determined from the number of eggs produced during the incubation period. Toxin content of *A. fundyense* used in this experiment was measured by HPLC in our laboratory (Oshima *et al.,* 1989; Colin and Dam, 2002a).

In order to examine individual variability, we measured during the fifth generation the ingestion rates of seven individual copepods from each line fed 150 μg CL^{-1} of either toxic *A. fundyense* or the non-toxic alga *Tetraselmis* sp. We employed the same procedure as described above, except that only one female copepod was placed into each 140-mL bottle.

Results

Life-history experiments

We observed reductions in the survival, growth and fecundity of the naïve *Acartia hudsonica* from NJ reared on the diet containing toxic *A. fundyense* (i.e. NJ-treatment copepods) that were consistent with the hypothesis that toxic *A.fundyense* reduces the demographic traits of copepods from the naïve population to a greater extent than those from the historically exposed population. The survival, l_x, of the NJ-treatment copepods was lower than that of the NJ-control and ME-treatment copepods (Fig. 3a; Table 1; Gehan–Wilcoxon non-parametric test for censored data, $p < 0.05$). Hazard plots show that the NJ-treatment copepods were most at risk of dying between days 6 and 20, during the copepodite stages (Fig. 3b). In contrast, the adult survival of the NJ-treatment copepods was not less than the NJ-control copepods (Fig. 4a; Table 2; comparison of NJ treatment to NJ control and ME treatment, Gehan–Wilcoxon non-parametric test for uncensored data, $p > 0.05$); therefore, adult survival was not affected by the presence of toxic *A. fundyense* in the diet.

Similarly, body length of the NJ copepods was reduced when their diet included toxic *A. fundyense* (Fig. 5a; comparison of NJ-treatment to NJ-control copepods, repeated measures ANOVA, df = 1, $p = 0.0023$). As a result, copepodites in the treatments were smaller than those in the controls after day 10 of the experiment (Fig. 5a; ANOVA between cohorts for specific days, df = 4, $p < 0.02$). Mature females from the NJ-treatment cohorts were also smaller in size than females from the NJ-control and ME-treatment cohorts (Table 3; Tukey–Kramer post hoc test, $p < 0.05$).

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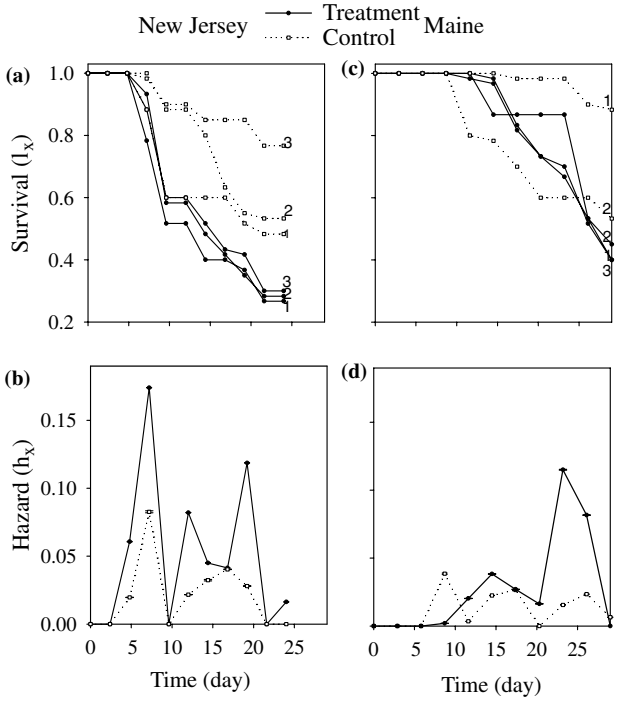

Figure 3. Survival and hazard plots throughout the whole life of New Jersey (a and b) and Maine (c and d) *Acartia hudsonica* populations fed diets with (treatment) and without (control) toxic *Alexandrium fundyense*. Survival plots show the survival of each of the triplicate cohorts for each population and diet (numbered 1–3, except ME-control where there are only two replicate cohorts). See Table 1 for statistical relationships among survival curves. Hazard plots represent the mean hazard coefficient of triplicate cohorts (standard error bars shown, $n = 3$, except $n = 2$ for ME-control). The hazard plots illustrate the probability of dying at different times.

Table 1. Gehan–Wilcoxon test results of survival data from the whole life experiment. Arrow indicates whether the survivorship of the cohort indicated in the column is greater (up arrow) or less (down arrow) than that of the cohort indicated in the row.[1] Blank spaces indicate differences were not significant

	NJ Control			ME treatment		
	1	2	3	1	2	3
NJ treatment 1	* ↑	*** ↑	*** ↑	*** ↑	*** ↑	*** ↑
NJ treatment 2		** ↑	*** ↑	*** ↑	*** ↑	*** ↑
NJ treatment 3		** ↑	*** ↑	*** ↑	*** ↑	*** ↑
ME control 1	*** ↓	** ↓	* ↓	** ↓	** ↓	** ↓
ME control 2			* ↓			

[1] Significant differences between cohorts (indicated as column and row titles) are indicated by asterisks (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$).

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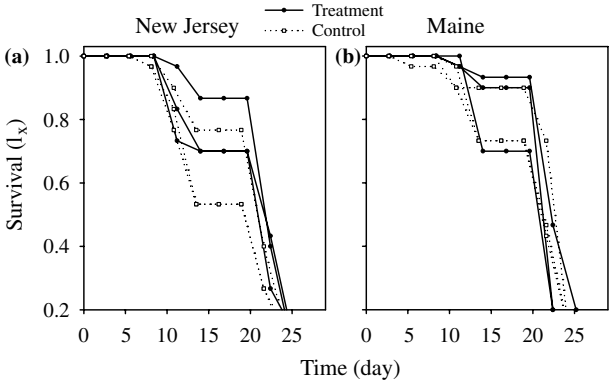

Figure 4. Survival plots of adult New Jersey and Maine *Acartia hudsonica* populations fed diets with (treatment) and without (control) toxic *Alexandrium fundyense*. Survival plots show the survival of each of the triplicate cohorts for each population and diet (numbered 1–3, except ME-control where there are only two replicate cohorts). See Table 3 for statistical relationships among survival curves.

Table 2. Gehan–Wilcoxon test results of survival data from the adult survival experiment. Arrow indicates whether the survivorship of the cohort indicated in the column is greater (up arrow) or less (down arrow) than that of the cohort indicated in the row.[1] Blank spaces indicate differences were not significant

	NJ Control			ME treatment		
	1	2	3	1	2	3
NJ treatment 1				* ↓		
NJ treatment 2	* ↓	* ↓				
NJ treatment 3						
ME control 1	* ↓	** ↓	* ↓		** ↓	* ↓
ME control 2						
ME control 3						

[1] Significant differences between cohorts (indicated as column and row titles) are indicated by asterisks (* $p < 0.05$, ** $p < 0.001$)

322 In addition to reduced body length, the fecundity, m_x, of the NJ-treatment
323 copepods was lower than the NJ-control copepods (Fig. 6a and b; ANOVA,
324 $df = 1$, $p < 0.005$). Since the age-specific fecundity measured on the two
325 separate days did not differ significantly within cohorts (ANOVA, $df = 1$, $p >$
326 0.05), we pooled the days to make the comparisons between cohorts types.
327 Of the life-history traits examined, the development rate of NJ-treatment
328 copepods, which was measured as the median development times (ANOVA
329 comparing median development times, $df = 1$, $p > 0.3$) and is illustrated in
330 Fig. 5b, was not affected by toxic *A. fundyense*.

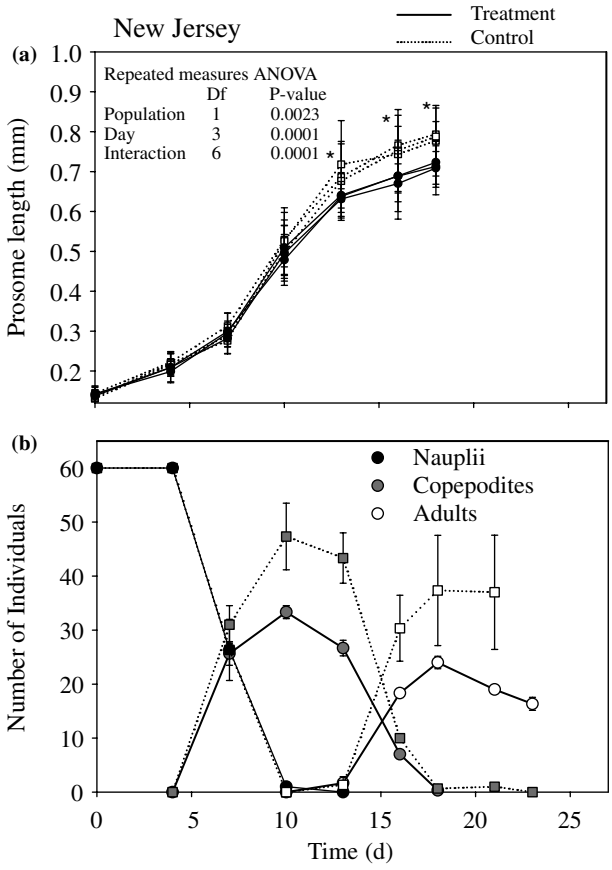

Figure 5. (a) Mean prosome length of individuals from each New Jersey cohort (control, dotted line; treatment, solid line) over time. Error bars represent standard errors of the means. Asterisks indicate significantly different lengths between control and treatment cohorts (Tukey–Kramer post hoc test, $p < 0.05$). (b) Mean number of individuals from the triplicate control (dotted line) and treatment (solid line) New Jersey cohorts that are at a particular life stage (nauplius, copepodite, adult) versus time. Treatment cohorts were reared with toxic *Alexandrium fundyense* present in their diets whereas controls were not.

331 In contrast to the naïve copepods, the historically exposed copepods from
332 Maine were not affected by the presence of toxic *A. fundyense* in their diet.
333 Their survival (whole life survival, Fig. 3c; Table 1; adult survival Fig. 4b;
334 Table 2, Gehan–Wilcoxon non-parametric test, $p > 0.05$), fecundity (Fig. 6,
335 ANOVA, $df = 1$, $p > 0.05$), development rate (Fig. 7a; Table 3, ANOVA
336 comparing median development times, $df = 1$, $p > 0.1$) and body length
337 (Fig. 7b, Repeated measures ANOVA, $df = 1$, $p = 0.8$) did not significantly
338 differ from the Maine cohorts reared on the control diet.

Table 3. Mean life-stage duration and size at maturity of treatment and control cohorts from whole life experiment. Standard deviation (n = 3, except n = 2 for ME-control) is given in parentheses. Asterisks indicate significant difference between control and treatments within a population.[1] Stage duration refers to the cumulative time in the copepodite or adult stage

	Cohort treatment			
	ME control	ME treatment	NJ control	NJ treatment
Stage dyration (d) Copepodie	11.8 (1.04)	12.8 (0.45)	6.9 (0.30)	7.1 (0.07)
Adult	20.9 (0.02)	21.3 (0.29)	15.2 (0.06)	15.2 (0.12)
Size at maturity (cm)	0.78 (0.04)	0.82 (0.05)	0.83 (0.06)	0.74* (0.06)

[1] Tukey–Kramer post hoc test, $p < 0.05$.

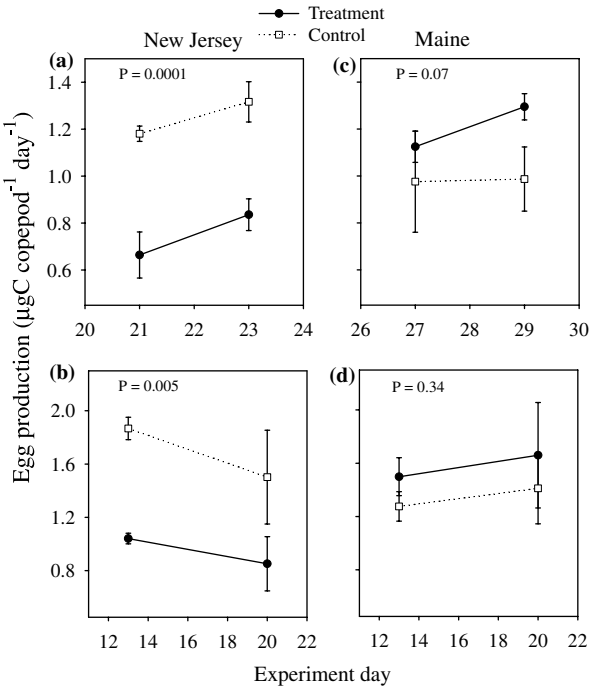

Figure 6. Mean egg production rates of adults from triplicate New Jersey (a and b) and Maine (c and d) cohorts measured on two different days during whole life (a and c) and adult (b and d) experiments. Treatment (solid lines) cohorts were reared with toxic *Alexandrium fundyense* present in their diet whereas controls (dotted lines) were not. Probability p values from one-way ANOVAs (egg production rates for the 2 days were pooled) comparing control versus treatment cohorts are indicated. Error bars represent standard errors of the means (n = 3).

339 In summary, the lower survival, somatic growth, size at maturity and
340 fecundity of copepods from the naïve population exposed to toxic *A. fundy-*
341 *ense*, relative to the historically exposed population, confirm our hypothesis

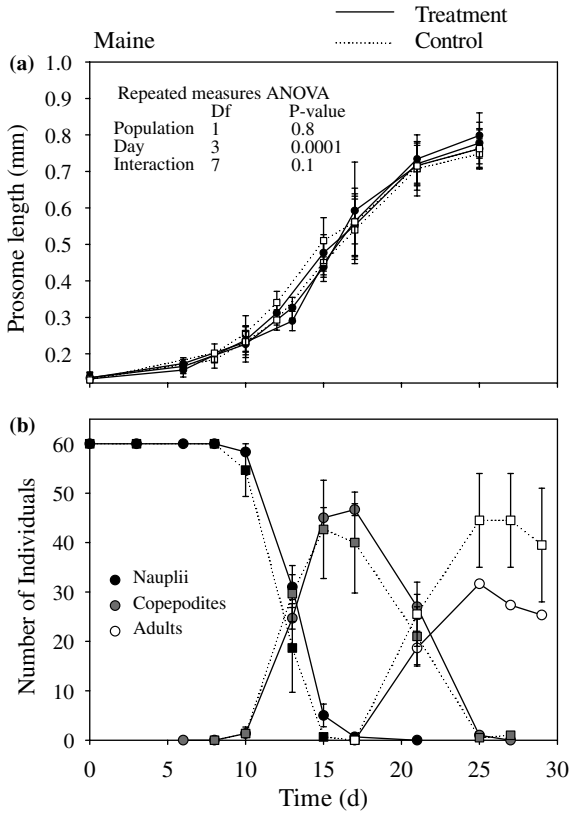

Figure 7. (a). Mean prosome lengths of individuals from each Maine cohort (control, dotted line; treatment, solid line) over time. Error bars represent standard errors of the means. (b) Mean number of individuals from the triplicate control (dotted line) and treatment (solid line) Maine cohorts that are at a particular life stage (nauplius, copepodite, adult) versus time. Treatment cohorts were reared with toxic *Alexandrium fundyense* present in their diet whereas controls were not.

that the effects of the toxic dinoflagellate on copepod life-history traits are related to copepod exposure history.

An interesting point is that we observed significant differences in the sex ratios (males/females) between the control cohorts and the cohorts fed *A.fundyense* for the Maine population. The Maine cohorts fed *A. fundyense* had a lower percentage of males at the end of the experiment on day 29 than the control cohorts (control = 52.1% vs. *Alexandrium* = 30.3%; single ANOVA for arcsine transformed percentages, df = 1, $p = 0.029$). We observed the same effect on the sex ratios for the New Jersey population (control = 47.8% vs. *Alexandrium* = 36.7%; single ANOVA for arcsine transformed percentages, df = 1, $p = 0.034$). Thus, it appears that the presence of *A. fundyense* in the diet skews sex ratio towards females.

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Table 4. Mean (standard deviation) fitness estimate of triplicate New Jersey (NJ) and Maine (ME) cohorts calculated using the age-class and stage-class models. $N = 3$ for Maine and $N = 2$ for new Jersey. Asterisks indicate a significant difference between the control and treatment cohorts within each copepod population[1]

Cohorts	Age-class	Stage-class	Stage-class (with adult data)
NJ copepods			
Control	1.56 (0.05)	1.32 (0.04)	1.30 (0.03)
Treatment	1.35 (0.02)*	1.23 (0.01)*	1.23 (0.02)*
ME copepods			
Control	1.41 (0.01)	1.26 (0.01)	1.24 (0.03)
Treatment	1.39 (0.02)	1.25 (0.03)	1.25 (0.01)

[1] Mann–Whitney *U*-test, $p < 0.05$.

Consistent with our hypothesis, every estimate of fitness (λ) of the naïve copepods from New Jersey fed the diet containing toxic *A. fundyense* was lower than the naïve copepods fed the control diet (Table 4; Mann–Whitney *U*-test, $p < 0.05$). In contrast, no differences in any of the fitness estimates were observed between the treatment and control cohorts of the Maine copepods (Mann–Whitney *U*-test, $p > 0.05$).

The historically exposed ME-control copepods exhibited reduced life-history traits compared to the NJ-control copepods. These include: longer development times (Table 3; ANOVA comparing median development times, $df = 1$, $p < 0.003$), smaller mature females (Table 3; Tukey–Kramer post hoc test, $p < 0.05$) and lower fecundity in the whole life experiment (Fig. 6c; ANOVA, $df = 1$, $p = 0.03$). However, these differences did not result in significantly lower population growth rates estimates for the ME-control cohort (Table 4; Mann–Whitney *U*-test, $p > 0.05$).

The replicates for ME- and NJ-control copepods and ME- and NJ-treatment copepods were placed in different buckets. With this design a bucket effect is possible and may confound the results. However, as just mentioned, the treatment did not significantly affect ME survival, growth, fecundity, development rate and fitness but did significantly reduce NJ survival, fecundity, growth and fitness. These interactions between population and treatment suggest that there was no bucket effect.

Genetic selection experiment

Both the ingestion and the egg production rates of the *Alexandrium* line copepods fed toxic *A. fundyense* were significantly greater than the control line copepods by the third and second generation, respectively (Fig. 8; *t*-test, $df = 1$, $p < 0.01$), and remained significantly greater for the remaining generations of the experiment.

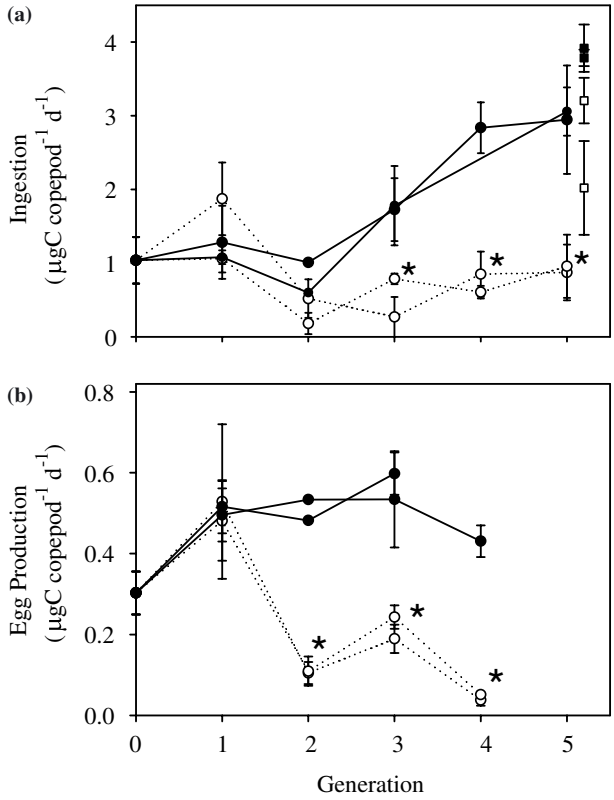

Figure 8. Ingestion (a) and egg production (b) rates of adult *Acartia hudsonica* from the *Alexandrium* (filled circles) and control (circles) lines fed 150 µg CL^{-1} toxic *Alexandrium fundyense* at consecutive generations. Asterisks indicate when the ingestion and egg production rates of copepods from the *Alexandrium* lines were significantly greater than the control lines (*t*-test, $p < 0.01$). Duplicate lines did not significantly differ (*t*-test, $p > 0.1$); therefore, the lines were pooled for comparisons between *Alexandrium* and control. The change in the ingestion and egg production rate of the control line among generations is most likely attributable to the variability in the cell toxicity of *Alexandrium* sp. at the time of each experiment (mean = 12.9 pg STXeq. Per cell ± 4.2 s.d.; minimum toxicity = 7.5 pgSTXeq. cell during first generation; maximum toxicity = 18.0 pg STXeq. per cell during third generation). Light and dark squares are the ingestion rates of the control and *Alexandrium* line copepods, respectively, fed 150 mg CL^{-1} *Tetraselmis* sp. during the fifth generation.

381 The ingestion and egg production rates of copepods in the control line
382 decreased over time, with the maximum ingestion and egg production rates
383 observed during the first generation. This is most likely attributable to the
384 differences in the mean toxicity of the *A. fundyense* cells among generations.
385 Toxicity of *A. fundyense* changed as a function of the conditions in the stock
386 cultures (e.g. nutrient availability and cell density), which were impossible to
387 keep constant throughout the 8-month experiment. The lowest toxicity of

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388 7.5 pg STXeq. per cell was observed during the first generation which corre-
389 sponds to the maximum ingestion and egg production rates in the control line.
390 While the toxicity was at least twice as high for the other generations in which
391 it was measured (14.9, 18.0 and 12.9 pgSTXeq. per cell for generation 0, 3 and
392 4, respectively).

393 The ingestion rates of individual copepods in the *Alexandrium* and control lines
394 fed the non-toxic flagellate *Tetraselmis* sp. were measured during the fifth gen-
395 eration. There was no difference in the ingestion rate of *Tetraselmis* sp. between
396 copepods in the *Alexandrium* an the control lines (Fig. 8; replicate copepod lines
397 were pooled [single ANOVA between replicate lines, $p > 0.1$]; single ANOVA
398 between *Alexandrium* and control lines, $p = 0.22$). However, individual cope-
399 pods in the *Alexandrium* line continued to ingest more toxic *A. fundyense* than
400 those in the control line in this generation (t-test, df $= 1$, $p < 0.01$).

401 In summary, the results of the genetic selection experiment are consistent
402 with the hypothesis that resistance to toxic dinoflagellates can evolve in pop-
403 ulations of marine copepods via natural selection. As shown here, the rate of
404 evolution can be quite rapid.

405 Discussion

406 While it has been previously shown that the effect of toxic *Alexandrium* spp. on
407 adult copepod ingestion and egg production is related to the exposure history
408 of the region from which the copepods originate (Colin and Dam 2002a, b), to
409 be able to attribute these differences to natural selection, such effects must
410 result in fitness differences among populations. The results from the present
411 study show that toxic *A. fundyense* affect the demographic traits of the naïve
412 copepod population from New Jersey, effectively reducing population fitness.
413 In contrast, such fitness reduction is not evident in the historically exposed
414 population from Maine. In addition, the results from the laboratory genetic
415 selection experiment provide strong evidence that *A. fundyense* acts as a
416 selective pressure that can affect rapid evolutionary change in the copepod
417 populations. Together, these studies support the hypothesis that the Maine
418 copepod population has evolved resistance to toxic *A. fundyense* by natural
419 selection.

420 *Effects on life history traits*

421 Our results demonstrate that the effects of toxic *A. fundyense* on the life-
422 history traits and population fitness of *Acartia hudsonica* vary geographically
423 among copepod populations. Lower survival, growth, size at maturity and

fecundity resulted when copepods from the naïve (New Jersey) population were reared with a diet containing toxic *A. fundyense*. Consequently, the fitness of the naïve population was reduced in the presence of toxic *A. fundyense*. In contrast, the demographic traits or fitness of the exposed population from Maine were not affected by toxic *A. fundyense*. Not only are these findings consistent with previously reported differences on the effects of toxic *A. fundyense* on adults of geographically distinct populations of *Acartia hudsonica* (Colin and Dam, 2002a), but also with the hypothesis that differences between populations are due to local adaptation in the copepod population from Maine.

Reduced feeding activity caused by the neurotoxic effects of *A. fundyense* probably explains most of the fitness reductions we observed in the cohorts from New Jersey. Colin and Dam (2003) found that toxic *A. fundyense* physiologically incapacitated non-resistant adult *Acartia hudsonica*, reducing their ability to feed effectively: diets containing only 20% toxic *A. fundyense* (by carbon, $\sim$50 μg CL^{-1}) reduced the total ingestion rate of NJ *Acartia hudsonica* to near zero within 12 h of exposure. In our experiment, the treatment diet consisted of 25% *A. fundyense* and was provided at a higher concentration. Therefore, it is likely that the feeding activity of the NJ copepodites in the treatment was severely reduced and, consequently, some copepodites may have been near starvation. Juvenile copepod stages have been shown to be more prone to starvation than adults (Tsuda, 1994; Lopez, 1996). Therefore, we would expect higher mortality in the copepodite stages feeding on toxic *A.fundyense* than in adults.

The demographic traits and fitness estimates of the ME copepods demonstrated that they were resistant to the effects of toxic *A. fundyense*. Other work has shown that adult female *Acartia hudsonica* from the same ME population are resistant to the toxic incapacitating effects of *A. fundyense* on feeding (Colin and Dam, 2002b). Toxin resistance in animals is mediated through different mechanisms: behavioral avoidance of toxic foods, increased rates of metabolic breakdown of toxins or decreased sensitivity to toxins (Taylor, 1986). If copepods are able to behaviorally identify and avoid toxic *A. fundyense,* they would either cease feeding activity when fed the alga as a sole food diet, select against the alga or, as is often observed, resume normal feeding when given a mixed diet (Colin and Dam, 2002b). However, female *Acartia hudsonica* from Maine ingest toxic *A. fundyense* at high rates regardless of whether it is provided as a sole or mixed food (Colin and Dam, 2002b). Similarly, Teegarden *et al.* (2001) found that *Acartia hudsonica* from Casco Bay, ME fed readily on toxic *A. fundyense* in natural algal assemblages. Thus, their resistance is not through avoidance of *A. fundyense*. Whether resistance of *Acartia hudsonica* is due to a metabolic mechanism to increase toxin breakdown or to reduced sensitivity to toxins is still an open question.

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Resistance may exert a fitness cost to individuals when the environment is free of the agent that induced the evolution of resistance (e.g. Luoma, 1977; Klerks and Levinton, 1989). The cost of resistance has implications for the interpretation of the experiments of this study. For instance, if the cost of resistance against *A. fundyense* was onerous, then one would expect selection against resistant individuals reared for many generations without *A. fundyense*. Hence, differences in the performance of the copepods from Maine fed diets with and without *A. fundyense,* which were done after 11 generations, could have been more pronounced if the experiments had been carried out a few generations earlier. In principle, we can examine costs of resistance by comparing the demographic traits and fitness of the historically exposed ME-control population to the naïve NJ-control population. If resistance has a cost, the ME population should have lower growth rate than the NJ population in an *Alexandrium*-free diet. Although the NJ-control copepods had higher demographic parameters and fitness estimates than both the control- and ME-treatment copepods, the fitness differences were not significant between the control ME and NJ cohorts (Table 4). Thus, the available evidence is not consistent with the idea that the cost of resistance is high. This is also consistent with how fast resistance evolved in the New Jersey experiment during the genetic selection experiment. However, two issues confound the interpretation of the life-history study in the context of the cost of resistance. First, the small sample size limited the statistical power of the comparison between the control Maine and New Jersey populations. Thus, it is possible that with a larger sample size, we could have indeed found significant differences between the two control populations. Second, even if these differences were found, we would have had to rule out that they were not due to cogradient physiological variation in copepods originating from different latitudes resulting from an adaptation to different temperatures (Lonsdale and Levinton, 1985; Conover and Schultz, 1995). For instance, depending on the experimental temperature, the development rate of a single copepod species has been shown to both increase and decrease among copepod populations from locations increasing in latitude from Maryland to Maine, USA (Lonsdale and Levinton, 1985). A more thorough study examining the effects between populations at different temperatures is needed to determine the causes of the fitness differences between the ME and NJ copepods.

Fitness and natural selection

Natural selection is the primary mechanism by which pelagic marine populations with high dispersal such as copepods can become genetically distinct (Hilbish, 1996). The presence of toxic *A. fundyense* in the diet of the NJ copepods clearly induced demographic changes (e.g. slower growth, lower

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survival and fecundity). These demographic effects along with the high genetic variation in marine copepod populations (Tepper and Bradley, 1989; Caudill and Bucklin, 2004) would likely cause natural selection. The introduction of toxic algae into freshwater systems has been shown to cause rapid evolution (decades time-scale) in *Daphnia* sp. populations (Hairston *et al.,* 1999). The results from the genetic selection experiment presented here suggest that toxic *A. fundyense* could cause rapid evolution in copepods.

The genetic selection experiment demonstrated that ingestion and egg production (a fitness trait) of adult females from a naïve *Acartia hudsonica* population fed toxic *A. fundyense* can be significantly improved when reared for only three generations in the presence of the toxic dinoflagellate (Fig. 8). As a result of the identical rearing conditions among cohorts, we can attribute the differences between the control and *Alexandrium* lines to genetic differentiation among cohorts and, thus, this change presumably occurred as the result of genetic selection within the copepod lines.

We recognize that the rate of evolution in our selection experiment is highly atypical and likely the result of an extreme population bottleneck. Nonetheless, this experiment demonstrates that toxic *A. fundyense* acts as a selection agent on populations of *Acartia hudsonica*, and that the rate of selection is potentially fast. From the life-history experiments, we can make a first order estimate of a typical time for a naïve *Acartia hudsonica* to become resistant. First, we can project the turnover of resistant versus non-resistant genotypes in a naive population, such as the NJ population, based on our estimates of finite population growth rate (for NJ-control, $\lambda = 1.56$, for NJ-treatment, $\lambda = 1.36$). We must assume that both resistant and non-resistant genotypes are present in the New Jersey copepod population and that the resistant individuals exhibit life-history traits and population growth rates similar to the control copepods when feeding on *A. fundyense*. Then, we will assume that $N_n = N_0\lambda^n$ (where N is the number of individuals and n is the generation) and that, partly based on the results from the genetic selection experiment, the N_0 proportion for the resistant : non-resistant individuals is 50 : 50. Accordingly, after 5, 10 and 20 generations we would expect there to be 2, 4 and 18 times more resistant copepods, respectively than non-resistant individuals in the population. This estimate demonstrates that with four to seven generations per season (Mauchline, 1998), the New Jersey copepod population would be dominated by resistant genotypes after only a few seasons of exposure to toxic *A.fundyense* blooms. However, given that *Acartia* appears to have geographically distinct populations (McAlice, 1981; Caudill and Bucklin, 2004), this assumption is probably not in gross error.

The results of this study are germane to management and control of spreading toxic algal blooms. It has been hypothesized that toxic dinoflagellate blooms occur because these phytoplankters have developed allelopathic antipredatory

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548 mechanisms that effectively counteract their low intrinsic growth rates
549 (Smayda, 1997). However, rapid local grazer adaptation would then mean that
550 grazing control is possible. The evolved resistance in the northern copepod
551 populations translates to a higher grazing pressure on growing toxic dinofla-
552 gellate populations that can effectively keep the blooms in check. Thus, the
553 adaptive evolution of zooplanktonic grazer populations to toxic algae may be
554 an important feedback mechanism in marine systems (Hairston *et al.,* 1999).
555 This might make the adaptive evolution of zooplanktonic grazer populations
556 an important feedback mechanism in marine systems (Hairston *et al.,* 1999)
557 enabling systems to cope with the introduction of toxic algae. Therefore, it is
558 essential to understand the role of evolutionary responses of grazer populations
559 to toxic algae in order to predict the ecosystem-level impact of spreading
560 harmful algae.

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