

The effects of the toxic dinoflagellate *Alexandrium* on feeding, reproduction and mortality of the copepod *Acartia*: A systematic review employing weighted linear models

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ARTICLE INFO

Editor: Dr. C. Gobler

Keywords:

Alexandrium

Acartia

Systematic review

PST

Adaptation

ABSTRACT

The study of interactions between copepods of the genus *Acartia* and toxic dinoflagellates of the genus *Alexandrium* has been an important topic during the last four decades. Feeding behavior and physiological responses of copepods have been studied in laboratory and field experiments, sometimes with contradictory results. More recently, an evolutionary adaptive mechanism leading to enhanced tolerance of *Alexandrium* toxins in a population of *Acartia* experiencing chronic exposure to these dinoflagellates has been reported. In the present work, we collected data from the existing studies on the effects of *Alexandrium* on feeding, reproduction and mortality of *Acartia*. With these data, we performed a systematic review consisting of a secondary analysis employing general or generalized linear models, weighting data from different studies by the reciprocal of their standard deviation. Our first aim was to overcome shortcomings of individual studies: limited ranges of the variables and overlooked variables (experiment length, population adaptation). These shortcomings could have led to inconsistent conclusions by missing heterogeneous patterns in copepod responses and in the interactions between variables. Our second aim was to test the enhanced physiological performance of chronically exposed relative to naïve copepod populations over a wide geographic range. We found that the feeding rate is enhanced by increased food biomass, irrespective of the food type. Toxins do not have a clear effect on egg production and have a bi-phasic effect on egg hatching success, which was negative above a specific threshold. Toxins also increased mortality. Experiment length had a positive effect on egg production and negative on egg hatching. Naïve copepod populations showed consistently lower ingestion of *Alexandrium* and egg hatching rates, thereby supporting the spread of the aforementioned mechanism across populations over a wide geographic range.

1. Introduction

During the last four decades, research on the phenomenon of harmful algal blooms has shown major advances. Their study has been approached from different fields of biology and several sub-topics of study have emerged; among these, the interaction between the harmful microalgae and meso-zooplankton grazers. This interaction was seen as a key step for understanding the fate of microalgae toxins in the trophic chain, since meso-zooplankton constitute a link to higher trophic levels. In planktonic and some benthic marine ecosystems, where microalgae dominate net primary productivity (NPP), more than 40 % of it is grazed

(based on a compilation of studies across marine ecosystems by Duarte and Cebrián, 1996). Among the grazers, meso-zooplankton is the group that globally consumes the most. In a global analysis of marine ecosystems, Calbet (2001) estimated that meso-zooplankton consume ~12 % of total oceanic primary productivity. Apart from being a critical link in the fate of microalgae toxins in the trophic chain, the relationship between harmful microalgae and meso-zooplankton also has strong implications in plankton community structure and function, as well as in top-down and bottom-up regulation processes.

Dinoflagellates of the genus *Alexandrium* are typical causal agents of harmful algal blooms with production of paralytic shellfish toxins (PST),

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<https://doi.org/10.1016/j.hal.2024.102659>

Received 15 February 2024; Received in revised form 14 May 2024; Accepted 24 May 2024

Available online 25 May 2024

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which can reach humans through the ingestion of contaminated seafood. Calanoid copepods usually contribute to the major fraction of pelagic meso-zooplankton biomass, *Acartia* being one of the most representative genera of that order. This size group can graze heavily on dinoflagellates (Calbet et al., 2003). There is no empirical evidence that this grazing could substantially reduce dinoflagellate populations during blooms (but see S.P. Colin and Dam, 2007, for modeling evidence for the opposite). Some species of dinoflagellates, such as *Karlodinium veneticum* were found to produce feeding deterrent compounds that reduce copepod feeding rates (Waggett et al. 2008). However, this was never found for *Alexandrium*. In any case, feeding of *Acartia* species on toxic dinoflagellates constitutes a main pathway for PST toxins along the trophic chain (Calbet et al., 2003; Tan et al., 2007).

All this has led to the study of *Acartia* and *Alexandrium* interaction being highly prolific, resulting in the publication of several works based on laboratory and field experiments, with the focus on their ecological relationships, sometimes with inconsistencies in the conclusions regarding *Acartia* feeding behavior and the physiological effects of toxins (Turner and Tester, 1997).

One of the aspects researchers were more focused on was the ability of these zooplankton grazers to select food particles. Copepods of the genus *Acartia* exhibit a bi-modal feeding behavior, which depends on prey size. They act either as generalist suspension feeders or selective ambushers (Kjørboe et al., 1996). Accordingly, when grazing on *Alexandrium* they would be expected to act as generalist suspension feeders. Researchers tested *Acartia* selective feeding behavior with field communities (Teegarden, 1999; Teegarden et al., 2001; Calbet et al., 2003) and with laboratory cultures, in order to determine specific abilities to discriminate microalgae according to size, nutritional properties or toxin content (Price and Paffenhöfer, 1985; DeMott, 1988; Barreiro et al., 2006; Henriksen et al., 2007). The results from these experiments were contradictory, sometimes supporting selective feeding behavior of *Acartia* against toxic *Alexandrium* (Teegarden, 1999; Guisande et al., 2002a; Senft-Batoh et al., 2015) and other times supporting non-selective behavior (Barreiro et al., 2006; A. 2007; Isari et al., 2013).

Another highlighted aspect of the *Acartia*-*Alexandrium* interaction was the general physiological performance of the copepods after ingesting PST toxins. Studies recording the effects on ingestion rates and mortality demonstrated negative effects (Frangópulos et al., 2000; Guisande et al., 2002b; Sopanen et al., 2011). The effects of toxic *Alexandrium* on individual copepod fitness (i.e. egg production, egg hatching, offspring viability) were also found to be negative (Dutz, 1998; Frangópulos et al., 2000; Guisande et al., 2002; Prince et al., 2006). Despite these reported associations between PST ingestion and physiological performance and fitness, there is still a lot of inter-experiment variation and even contradictory results. Some works reported effects on mortality, but not on ingestion rates (da Costa et al., 2002, 2008). Teegarden and Cembella (1996) found no physiological effects of toxic *Alexandrium tamarense* and *A. fundyense* on *A. tonsa*, despite copepods feeding on these dinoflagellates at high rates. Similarly, Turner and Anderson (1983) found no effects of *A. tamarense* on *A. hudsonica*, although in this case, copepods fed at relatively lower rates. Some authors claimed that these inconsistencies and disagreements could be due to the fact that grazer-phytoplankton interactions are highly species specific. However, in these cases only different populations of the same species or different species of the same genus were studied. Important factors that were overlooked in experimental designs could be heterogeneous responses to the effect of toxins across the range of toxin concentrations employed, the dilution effect due to the presence of non-toxic food, total duration of the experiments and the specific adaptation of the populations.

In more recent times, some authors explored the role of evolutionary adaptation in the response of copepod populations from Maine to their interaction with toxic dinoflagellates (Colin and Dam, 2005; S.P. 2007; Zheng et al., 2011). These works suggested that copepods that have experienced toxic dinoflagellates for several generations (chronically

exposed populations) would have developed enhanced tolerance to the effects of PSTs. This was shown in terms of ingestion rates, egg production, hatching success and survival, for which the individuals from chronically exposed populations could be not affected at all or significantly less than the naïve populations.

In the present work we aim to study the physiological effects of toxic *Alexandrium* on *Acartia* by means of a systematic quantitative synthesis that encompasses the entire ranges of the main variables at play: toxin concentration, non-toxic food supplied, toxic *Alexandrium* supplied, experiment length and the influence of the degree of adaptation of copepod populations to the toxic *Alexandrium*. For this purpose, we gathered the physiological variables most frequently analyzed (ingestion rates, egg production, egg hatching, and mortality) from the available literature and applied a secondary analysis. These variables were statistically modeled, using the general linear model and extensions, as a function of toxic *Alexandrium* and non-toxic food availability, PST toxin concentration, experimental length and adaptation of *Acartia* population to the presence of toxic *Alexandrium*. This approach allowed us to study those variables in a single statistical model, covering wider ranges than in any individual study, allowing us to detect heterogeneous responses across the range of the variables, interactions or co-linear effects among the variables in a wider range and to include previously overlooked variables (experiment length, chronically exposed versus naïve copepod populations). In addition, we will be testing whether the results first found by Colin and Dam (2002b, 2005) showing improved performance of chronically exposed populations, are spread across populations of these two organisms in a wider geographical range.

2. Methods

We employed copepod species of the genus *Acartia* as the target of our study. This choice was justified for two reasons: first, this genus is the most frequently studied and there are not enough studies on other genera to be included in a balanced meta-analytical approach. A search of the web of science employing the terms ‘copepod AND *Alexandrium*’ returned 40 studies employing *Acartia* and the next most frequent genus was *Eurytemora* with 9. However, not all of these studies met the requirements for selection for the present work (see below). With these conditions, it was not possible to include other genera in our analysis. Second, the functional traits related to the feeding ecology of the copepods (mainly the anatomy of the feeding appendages) are the same across species of the genus *Acartia*. For this reason, we can consider the genus as an homogenous entity and there is no need to test for differences between species.

Using the keywords *Acartia* AND *Alexandrium*, we searched Web of Science for experimental studies published from 1970 to present and in Google Scholar for any kind of study available. We selected those studies in which copepods were exposed to diets including dinoflagellate, while reporting values of any of the following variables, that we will use as dependent variables: copepod ingestion rates (total biomass, biomass of toxic *Alexandrium*, and biomass of non-toxic food), egg production rates, egg hatching rates and accumulated mortality. The ingestion rates were measured from different experimental designs: diets containing only the PST producer dinoflagellate from the genus *Alexandrium*, only non-PST producer species or strains of this genus, control diets employing non-toxic species, or mixtures of all paired combinations of the previous three. For mixtures, we gathered the ingestion rates of each separate kind of food. We standardized the measures of ingestion and egg production rates as units of biomass ingested per individual and eggs produced per individual and day.

The individual datasets gathered consisted of treatment level means of the response variables, with some estimate of deviation. All the software employed in the data extraction and analysis was from R (R Core Team, 2022).

The software employed to extract data graphically was the *meta-Digitise* R package. In this work, it was not possible to obtain a

dimensionless standardized measure of effect size, as required in meta-analytical approaches, and for this reason we needed to perform a secondary analysis. Effect sizes cannot be calculated since a reference control is not always employed, which very often would be technically not viable anyway. This is a different frame for systematic quantitative reviews, but it does not constitute a loss of data or quantitative accuracy since the independent variables to analyze are the same across the studies and we can obtain these variables in the same units.

Among our predictor variables, we had non-toxic food biomass available. As non-toxic food was supplied different species of microalgae. However, in our analysis, we did not differentiate between these species. We believe that this approach is suitable for two reasons. First because the non-toxic microalgae supplied were always a highly nutritional species, or a mix of two. And second, because many studies employed the same species (only 11 different species employed - two of them of the same genus - and 2 field assemblages, out of 20 studies and 166 sets of data with this variable).

In order to integrate data from all sources in a single analysis, sample size needs to be weighted, since it differs between studies. Because deviation is considered to be inversely related to sample size, we employed the inverse of standard deviation to weight individual datasets in order to account for the differences in sample size (Koricheva et al., 2013). The only exception for this was the response variable survival, reported as accumulated mortality, for which no estimate of deviation is possible.

We only selected those studies reporting estimates of available food as carbon and measures of PST toxin concentration per unit biomass of *Alexandrium*. These two variables were the numerical independent variables that we included in our statistical models. Dinoflagellate biomass per cell was either reported in the study or found from other studies for the same strain. In order to obtain the values of biomass per cell in species other than dinoflagellates, if not reported in the study, they were searched for in the literature. In some instances, it was not possible to obtain direct values of biomass, but it was possible to obtain measures of cell size, which were transformed into biomass using the standard conversions from Menden-Deuer and Lessard (2000), after approximating volume to the nearest geometrical shape (Hillebrand et al., 1999). PST content was always reported as pg or already as fmol. We included it in our analysis as fmol of saxitoxin, including all the gonyautoxins reported together.

The level of exposure of the copepods to the presence of the genus *Alexandrium* in their environment was introduced as a two-level categorical factor (chronically exposed population/naïve population). These two levels were established based on the existence of records of the recurrent presence of the genus *Alexandrium* in the grazers' environment. The population was assumed to be adapted or acclimated when chronically exposed. Chronical exposure is not a direct demonstration of adaptation, as we will explain in the discussion.

The level of exposure was the only fixed categorical factor in our statistical models. Due to the irregular distribution of replicates between studies, this factor was unbalanced. In order to account for this aspect, type II sum of squares was employed in the analysis of variance performed (Langsrud, 2003). Other independent variables that we gathered were experiment duration and copepod population sampled. Copepod population sampled was intended to be included as a random factor, but in the end it was not possible due to the highly unbalanced number of replicates per population, and the low number of them (1–3) for several populations.

The final result of our selection was 28 studies, dating from 1996 to 2023. Table S1 shows the selected studies with the total data each one contributes to the analysis of the selected variables, copepod population of origin and its degree of exposure (and also whether adaptation was tested or not, and how) and the rejected studies, with the reasons for that rejection. In total, our literature search provided 207 sets of data for the analysis of ingestion rates of total biomass, 107 for the analysis of ingestion rates of toxic *Alexandrium* biomass, 166 for ingestion rates of non-toxic food biomass, 266 of egg production rates, 129 of egg hatching

rates and 40 of accumulated mortality.

The general approach for the analysis started with the exploration of relationships between variables and their dispersion. If the data were considered suitable, we fit a General Linear Model to each of the response variables, with all the independent variables recorded and using the inverse of the standard deviation as the weight (except for the accumulated mortality, see above). These models were implemented with the *lm* R function, from the *stats* package. Independent variables that are strongly collinear should be removed from the models, since a big portion of the variance that they help explain is redundant. With the purpose of selecting collinear variables we employed the index known as “variance inflation factor” (VIF) which measures the proportion of variance explained by each variable that is due to multi-collinearity. As a selection criterion, we employed the common practice of eliminating multi-collinear variables when their VIF value was > 4 . VIF calculation was implemented with the *vif* function from the *car* R package. Outliers in the response variable were detected with the Bonferroni test and removed. This test was implemented with the *outlierTest* function from the *car* R package. A model was considered optimal for further analysis when the residuals met the normality assumption (tested with the Kolmogorov-Smirnov test when $n > 100$, otherwise with the Shapiro-Wilks test). To achieve this normality, it was sometimes necessary to perform variable transformations and/or eliminate influential data. The optimal transformation for the response variable was performed with the *bestNormalize* function from the homonym R package or with the *boxcox* function from the *MASS* R package. Influential points in the response variable are data that show high residual values (thus far from the model fit) and high “leverage” (distance to average values from each independent variable). These data were eliminated after being detected with the algorithm of the function *influencePlot* from the *car* R package.

For those response variables with an obvious excess of dispersion when compared to a Normal distribution, a Generalized Linear Model was fitted. In these models, we selected between Poisson or Negative binomial as potential distributions for the residuals. These two distributions can fit models with an excess of dispersion of the response variable, in particular the negative binomial distribution. In order to select which of these two distributions is more suitable, it is necessary to perform an over-dispersion test. If the result of this test is significant over-dispersion, it will be necessary to employ the Negative Binomial distribution, otherwise the Poisson distribution. This test was performed with the *dispersiontest* function, from the *AER* R package. These models were fitted with the *glm* function for the Poisson models and the *glmmTMB* function for the negative binomial. These functions are from the *stats* and *glmmTMB* R packages, respectively. The effect of the independent variable “experiment length” was tested in two possible ways: as a standard predictor, or as an offset variable (either as its raw value or the inverse). An offset variable is defined as a variable representing exposure or measurement time, or population size from which the single values of the response variable were taken. Its coefficient in the model is constrained to be 1, so it will be directly interpreted as a rate. It is obvious that “experiment length” could be potentially treated as an offset variable, but it should be compared as well with its performance as a predictor. When this variable was non-significant, it was removed from the model.

In the final model versions, the test of significance for each independent variable was performed with Analysis of Variance for models fitted by least squares (models with Normal error distribution and without random factors) or Analysis of Deviance for models fitted by likelihood (for Generalized linear models with error distributions other than Normal or with random factors). These tests of significance were implemented with the *Anova* function from the *car* R package.

The effect of toxin concentration on egg production and egg hatching success showed a bi-phasic relationship. For this reason, its effect was analyzed with a segmented regression method. The segmented regression method detects thresholds that split the dataset into segments showing a distinct trend or relationship between the two variables. Then

a different regression will be fitted to each of these segments. A prior step to performing this regression was fitting a model removing toxin concentration and (in the case of egg hatching success) also population adaptation as predictors. Then, this model will miss the variance explained by these variables, which, if it is significant, will generate a pattern in the residuals. Then, we extracted the residuals of this model and performed the segmented regression between them and toxin concentration and, for egg hatching success, also population adaptation. First, the threshold of toxin concentration separating each segment (or phase) was determined with the *chngptm* function, from the homonym R package. Then, separate models were fitted to these residuals in the segments below and above the threshold.

3. Results

3.1. Ingestion rate of total food

For this response variable, the dataset of the fixed categorical factor population exposure consisted of 130 sets of data in the category ‘chronically exposed’ and 77 in the category ‘naïve’. Food biomass available (both from toxic *Alexandrium* or non-toxic food) had a positive significant effect, as well as PST toxin concentration in food (Table 1). The coefficient showed that naïve copepod populations had lower average ingestion rates than chronically exposed populations (Table 1). All these variables were found to be significant main effects in the ANOVA (Table 1). Fig. 1 shows the total ingestion rates with the model predictions for chronically exposed and naïve populations. We can see how chronically exposed populations have a higher intercept, but also a stronger response to the increase in food concentration.

3.2. Ingestion rate of *Alexandrium*

For this response variable, two transformations were needed, an initial square root transformation and then a Box-Cox transformation. 18 outliers were eliminated (16.8 %) and residual normality was obtained.

Table 1

Summary of the statistical models fitted and analysis of variance/deviance performed with all the response variables. The asterisks (*) indicate that the values shown for toxin concentration are from the main models, not from the segmented model (see text). For the population exposure, the coefficients are referred to the naïve populations. Experiment length was removed from the ultimate models when it was non-significant (n.s). *Alexandrium* carbon was removed from the model of accumulated mortality due to collinearity with toxin concentration (c.).

Model fit												
Independent variable	Total ingestion rate		Ingestion of <i>Alexandrium</i>		Ingestion of non-toxic food		Egg production		Egg hatching		Accumulated mortality	
	Coefficient estimate and p-value											
<i>Alexandrium</i> carbon ($\mu\text{g mL}^{-1}$)	1.5	< 0.001	1.1	< 0.001	-0.2	0.7	3.8	< 0.001	0.3	0.3	-	c.
Non-toxic species carbon ($\mu\text{g mL}^{-1}$)	1.4	< 0.001	-3.4×10^{-2}	0.5	1.4	< 0.001	2.6	< 0.001	4.1×10^{-2}	0.2	33.5	< 0.01
Toxin (fmol)	2.2×10^{-5}	< 0.01	1.2×10^{-5}	< 0.01	1.1×10^{-4}	0.8	$-1.2 \times 10^{-5*}$	0.5*	$-7 \times 10^{-5*}$	0.3*	1×10^{-3}	< 0.01
Experiment length (days)	-	n.s.	-	n.s.	-	n.s.	0.1	< 0.001	-8.6×10^{-2}	< 0.001	-	n.s.
Population exposure	-0.2	0.01	-0.3	< 0.01	-0.4	< 0.001	-1.1	< 0.001	-0.2	0.1	-7.5	0.3
Analysis of variance/deviance												
Independent variable	Total ingestion rate		Ingestion of <i>Alexandrium</i>		Ingestion of non-toxic food		Egg production		Egg hatching		Accumulated mortality	
	<i>F</i> / <i>chi</i> ² df model, df residual and p-value											
<i>Alexandrium</i> carbon ($\mu\text{g mL}^{-1}$)	39 _{1,202}	< 0.001	51.3 _{1,80}	< 0.001	0.2 _{1,155}	0.7	92.6 _{1,260}	< 0.001	0.9	0.3	-	-
Non-toxic species carbon ($\mu\text{g mL}^{-1}$)	87.9 _{1,202}	< 0.001	0.4 _{1,80}	0.5	40.1 _{1,155}	< 0.001	119.9 _{1,260}	< 0.001	1.5 _{1,123}	0.2	10.2 _{1,35}	< 0.01
Toxin (fmol)	8.4 _{1,202}	< 0.01	9.9 _{1,80}	< 0.001	0.1 _{1,155}	0.7	0.4 _{1,260*}	0.5*	0.9 _{1,123*}	0.3*	8.1 _{1,35}	< 0.01
Experiment length (days)	-	n.s.	-	n.s.	-	n.s.	35.9 _{1,260}	< 0.001	16 _{1,123}	< 0.001	-	n.s.
Population exposure	6.6 _{1,202}	0.01	11.1 _{1,80}	< 0.01	14.2 _{1,155}	< 0.001	52.6 _{1,260}	< 0.001	2.7	0.1	1.2 _{1,35}	0.3

For the fixed categorical factor population exposure, 54 data corresponded to the category ‘chronically exposed’ and 35 to the category ‘naïve’.

Alexandrium biomass available and PST toxin in food showed a significant effect, being positive in both cases (Table 1). The naïve populations showed significantly lower ingestion rates of *Alexandrium* (Table 1). *Alexandrium* biomass, PST toxin in food and population exposure were found to be significant main effects in the ANOVA (Table 1).

3.3. Ingestion rate of non-toxic food

This variable was square root transformed. After eliminating 6 (1.8%) outliers/influential data points, residual normality was obtained. For the fixed categorical factor population exposure, 110 data corresponded to the category ‘chronically exposed’ and 50 to the category ‘naïve’.

Non-toxic food biomass had a significant positive effect (Table 1). This variable and population exposure were the only ones significant as main effects in the ANOVA (Table 1). As for total food ingestion, the naïve populations showed significantly less ingestion of non-toxic food than the chronically exposed ones.

3.4. Egg production rate

This response variable presented large dispersion. For this reason, we initially fit a model with a Poisson error distribution, for which it was necessary to round the variable values to integers. Then, we performed an over-dispersion test, which showed significant over-dispersion ($p < 0.01$). For this reason, we fitted a model with a negative binomial distribution. For the fixed categorical factor population exposure, 146 data points corresponded to the category ‘chronically exposed’ and 120 to the category ‘naïve’.

An exploratory plot of the individual relationship between this response variable and the numerical predictors showed a winding shape

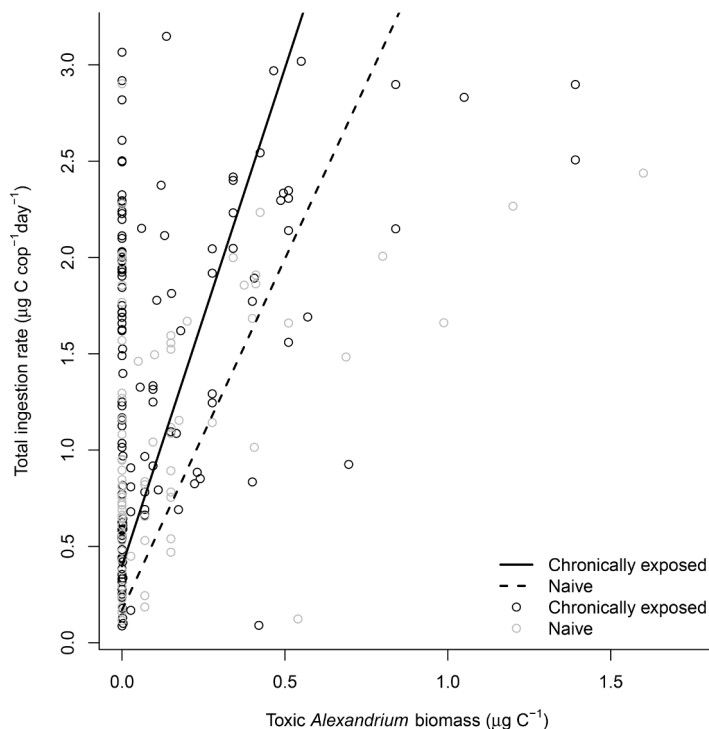


Fig. 1. Scatterplot of total ingestion rates versus the available biomass of toxic *Alexandrium* sp. Lines represent model predictions for the chronically exposed and naïve populations.

for the effect of toxin concentration (Figure S1). There was an initial phase in which the effect was not clear, and a second phase, after a certain threshold, showing apparently negative effect (Figure S1). For this reason, in addition to the model described before, we fitted a segmented regression model between the residuals of the main model (removing toxin concentration as predictor) and toxin concentration.

Our hypothesis was that toxin concentration in the global model is not significant due to this segmented relationship, apparently with different effects of toxin, which depend on the interval of data values. The global model cannot capture this bi-phasic effect of toxin concentration, and hence its residuals should still show a similar bi-phasic pattern related to toxin concentration. The initial step of the segmented model was to

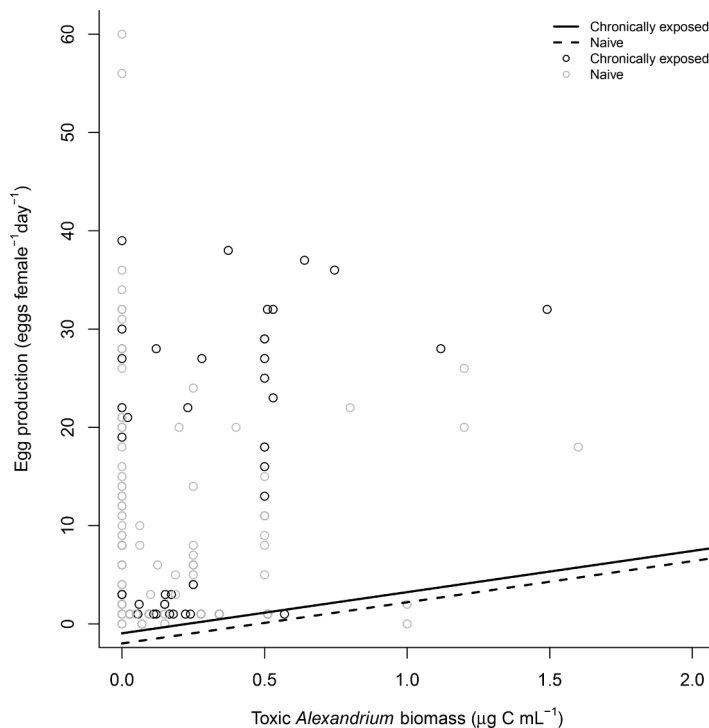


Fig. 2. Scatterplot of total egg production rates versus the available biomass of toxic *Alexandrium* sp. Lines represent model predictions for the chronically exposed and naïve populations.

estimate the threshold of toxin concentration separating the two phases observed. This threshold was 7325 fmol of PST, but with a very large 95 % confidence interval (± 5987 fmol). Then we performed a linear regression with the values on each side of the threshold, between the model residuals and both the toxin concentration and population adaptation. The results from these models showed, below the threshold, a significant negative effect of toxins (slope = -2.2×10^{-4} , p-value = 0.03) and above the threshold there was also a significant negative effect of toxin (slope = -2.1×10^{-5} , p-value < 0.001). The regression below the threshold was performed on 232 data points and for the regression above the threshold there was a total of 34. These results represent an unclear effect of toxins.

In the main model, non-toxic food biomass, *Alexandrium* biomass available, and experiment length had a significant positive effect, and the naïve populations showed significantly lower egg production rates (Table 1). In the Analysis of Deviance, all these variables were significant main effects (Table 1). The effect of toxins was overall negative, but because of its bi-phasic relationship, this model was unable to find it significant (Table 1). Fig. 2 shows data and model predictions for chronically exposed and naïve populations. In this case, naïve populations had lower intercept but the same slope, indicating that egg production rates responded in the same way to the experimental conditions.

3.5. Egg hatching success

This response variable also presented a large dispersion. We fit a model with a Poisson distribution, for which the values of the response variable were rounded to integers. Then, we performed an over-dispersion test, which showed significant over-dispersion ($p < 0.001$). For this reason, we fitted a model with a negative binomial distribution. We eliminated 2 outliers (1.7%). For the fixed categorical factor population exposure, 75 data corresponded to the category 'chronically exposed' and 52 to the category 'naïve'.

An exploratory plot of the individual relationships between this response variable and the numerical predictors showed a winding, or apparently segmented shape for the toxin concentration (Figure S2). There is an initial part in which the effect is apparently positive, and a second part, after certain threshold showing apparently negative effect (Figure S2). For this reason, in addition to the model described before, we fitted a segmented regression model between the residuals of the main model (removing toxin concentration and population exposure as predictors) and toxin concentration and population exposure. In this case, we included population exposure in the segmented regression because this variable was not significant in the global model (see below) which was not the case for egg production. If toxin concentration has a segmented effect, with different kind of relationships depending which interval the data comes from, the global model cannot capture this segmented effect of toxin concentration, and hence its residuals should show some pattern related to toxin concentration. And, maybe, population exposure is significant in some (or all) of these segments. The first step to build the segmented model was to estimate the threshold, which was 35 fmol of PST (no estimate of confidence intervals given). Then, we fit two linear models between toxin concentration, population exposure and the model residuals, one model below and other above this threshold. For the model below the threshold, there was a non-significant effect of toxins (slope = 0.007, p-value = 0.44) and a significantly positive effect of population exposure (slope for the naïve = -0.27 , p-value < 0.01). Above the threshold there was a significant negative effect of toxin concentration (slope = -1.3×10^{-4} , p-value < 0.01) and also a significant positive effect of population exposure (slope for the naïve = -0.3 , p-value < 0.01). The model below the threshold was performed on a total of 77 data points. The model above the threshold was performed on a total of 56.

In the main model, experiment length, with a negative effect, was the only variable found to be significant in the Analysis of Deviance

(Table 1). Fig. 3a shows data and model predictions as a function of experiment length (The most significant variable in the model, see Table 1). Fig. 3b shows the negative effect of toxin concentration in the simple linear regression fit to the residuals above the estimated threshold.

3.6. Mortality

For this response variable, according to the Variance Inflation Factor, the biomass of *Alexandrium* should be eliminated due to collinearity with toxin concentration. One outlier was eliminated (2.5%). For the fixed categorical factor population exposure, 20 datasets corresponded to the category 'chronically exposed' and 19 to the category 'naïve'.

Non-toxic food biomass available and PST toxin in food had a significant positive effect, and were significant main effects in the ANOVA (Table 1).

3.7. Summary of effects

Fig. 4 shows a forest plot of the standardized coefficients and their 95% confidence intervals for all the independent variables in all the statistical models. The strongest overall effects corresponded to the second-phase negative effect of toxin concentration in egg hatching (although with a large confidence interval). The rest of the effects were comparatively small, apart from the obvious positive effects of *Alexandrium* and non-toxic food biomasses on their respective ingestion rates. Toxin concentration also showed quite a strong positive effect in the ingestion of *Alexandrium*.

4. Discussion

All the variables analyzed showed very large dispersion. This was particularly obvious in the analysis of egg production rates and hatching success, for which a model with negative binomial distribution for the error was needed. This is very obvious in Figs. 1-3. This over-dispersion implies, statistically, that significant differences are more difficult to find, due to the larger error allowed for coefficient estimations. However, we observed that for several key independent variables, the significant effect was clear, being the regressions shown in Figs. 1-3 significant, despite of this over-dispersion. One of the sources for this over-dispersion that we could hypothesize would be the lack of estimation per replicate (or at least treatment level) of the food concentration provided. In many studies, authors report the food concentration as fixed values for all treatment levels and replicates. These 'fixed' values would correspond to their intended ones. However, the actual values may differ from the intended ones. Other studies in which those actual values are estimated and reported showed consistent clear differences in the food concentrations intended and the real ones. In our data sets, as a result of all this, for a single value of food concentration, we have a dispersion in the values of the response variable that might actually be due to an actual wider range than reported in the food concentrations provided.

The biomass of *Alexandrium* and non-toxic food had the strongest positive effect on the total ingestion rates. Between these two variables, as we can see in Fig. 4, the coefficient of the non-toxic food was slightly larger. Under most of the conditions from these studies, *Acartia* should behave as a generalist filter-feeder (Kiørboe et al. 1996) selecting particles based on size (Wilson, 1973). However, size could not be driving selection towards non-toxic food, since the non-toxic species are in the majority of cases smaller flagellates or diatoms (*Tetraselmis*, *Rhodomonas*, *Phaeodactylum*) in contrast to larger non-toxic dinoflagellates (*Prorocentrum*, non-toxic *Alexandrium*). Then, we should explain these results with the existence of some degree of selection against toxic food. This has occasionally been found in *Acartia* species (DeMott, 1988; Guisande et al., 2002a; Selander et al., 2006; Senft-Batoh et al., 2015).

There was a clear positive effect of PST toxins on total ingestion rates

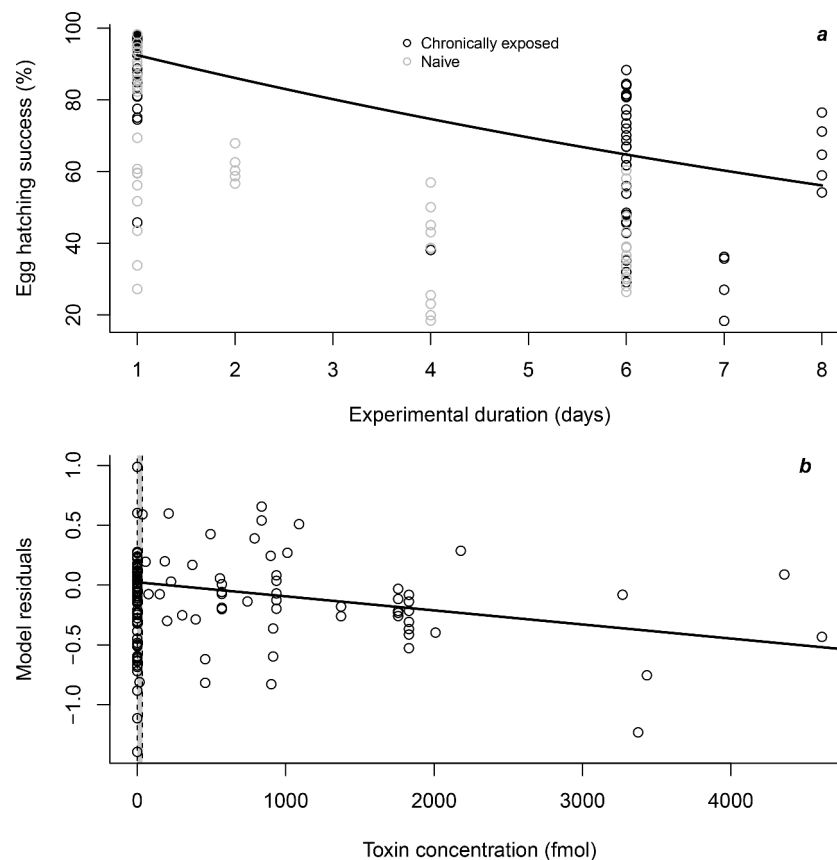


Fig. 3. **a:** Scatterplot of egg hatching success versus the most significant independent variable in the model (experimental length, see Table 1). Line represents model predictions. **b:** segmented linear regression model applied to the residuals of the main model. The grey rectangle delimits the data below the threshold in which toxin concentration has a non-significant effect. The line represents the regression segment above the threshold in which toxin has a significant negative effect.

and ingestion rates of *Alexandrium*. In these analyses, toxin concentration was not collinear with *Alexandrium* biomass. Then, this effect of toxin concentration suggests a positive feeding selection for the most toxic strains. This pattern could be, to some extent, driven by size selection, since the most toxic strains are usually larger in size. Overall, this analysis still has the shortcoming that, in most works, ingestion rates were only estimated in the short-term (except in Frangópulos et al., 2000; Guisande et al., 2002a, b; Barreiro et al., 2006, A. 2007). If, as we saw, PST toxins in the middle or long-term had a positive effect on mortality (Table 1, Fig. 4) they should be expected to negatively affect feeding rates at some point.

There does not seem to be an interaction between the availability of toxic *Alexandrium* and non-toxic food with respect to the ingestion rate of each kind of food. In both cases, the respective coefficients are far from being significant (Table 1) and proportionally very close to 0 (Fig. 4).

Overall, our results suggest a general pattern of weak selective behavior of *Acartia*, based positively on size and negatively on toxic content. With these results, we could contribute to the debate of whether *Acartia* can select based on more complex features of food, such as nutritional quality or toxicity, for which controversial results exist (DeMott, 1988; Guisande et al., 2002a; Selander et al., 2006; Barreiro et al., 2006; A. 2007; Isari et al., 2013; Senft-Batoh et al., 2015).

There were no strong effects detected on egg production (Fig. 4). It is remarkable that *Alexandrium* biomass had a clear positive effect over its whole range (Fig. S1). The effect of experiment length can be explained by the time lag of the response of egg production to food conditions. Copepods start the experiments producing eggs at rates that depend on the food conditions in the field in the days prior to the sampling. Then, they take several days to adapt their egg production rates to the food

conditions in the laboratory, which are in general more favorable. Although many studies do not estimate egg production rates on the first day of the experiment, work measuring this variable over the long-term showed clearly how the time lag to adapt to food conditions extends to 2–3 days (Frangópulos et al., 2000; Guisande et al., 2002a, b; Barreiro et al., 2006, A. 2007). Toxin had a negative but non-significant effect on egg production (Table 1, Fig. 4). We believe this result is consistent. The segmented regression showed significant negative relationships when fitting the models in two data intervals split by an estimated threshold (see text in Results). In our opinion this does not conform to a consistent negative effect. First because the threshold shows a wide confidence interval, and second, because there is no clear reason for the segmentation of two negative relationships that are unconnected to each other.

Egg hatching success was strongly affected by the negative second phase of its relationship with toxin concentration. The threshold for this negative effect was low. Previous work has already demonstrated that the effect of toxin on egg hatching is dose-dependent and not counteracted by the dilution effect of the presence of non-toxic food (Barreiro et al., 2006; A. 2007). The negative effect of experiment length is significant but not very strong (Table 1, Fig. 4). This effect could be due to stress conditions in most of the laboratory experiments or lack of specific nutrients that only shows up long-term.

The significant positive effects of toxins and non-toxic food increasing mortality were comparatively small (Fig. 4). This effect of non-toxic food enhancing mortality was somewhat unexpected. The explanations for this could only be speculative. A relatively feasible explanation could be that the elevated abundance of food employed in laboratory experiments, particularly in treatments with saturating food, could be causing some degree of anoxia in the experimental flasks during the dark. Another explanation could be nutrient deficiency or

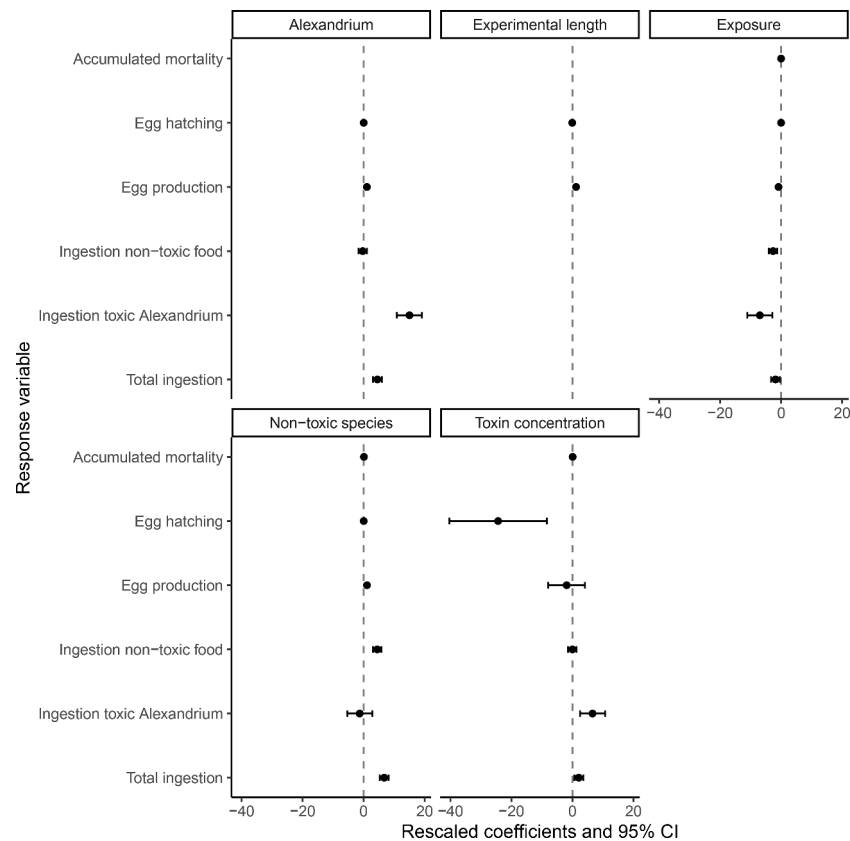


Fig. 4. Forest plot of standardized model coefficients and their 95 % confidence intervals. The coefficient standardization was performed relative to the scale of the corresponding independent variable. All coefficients are from the global models except the one for toxin concentration on egg hatching, which corresponds to the second segment of the relationship (values of toxin concentration above the threshold) the only one in which the effect of toxins was found to be significant (see Results).

unknown toxins present in the non-toxic food. However, these latter explanations are very unlikely, since non-toxic food controls are usually well known as being good food for copepods.

Our approach, by employing the whole range of values for the variables found throughout the literature, allowed us to make, for the first time, several inferences: first, the existence of a bi-phasic effect of toxins on egg hatching, with a threshold to induce the negative effect. This was an issue for which contradictory results existed (Teegarden and Cembella, 1996; Dutz, 1998; Frangópulos et al., 2000; Guisande et al., 2002; Prince et al., 2006). Second, the analysis of simultaneous ingestion rates of *Alexandrium* and non-toxic food over a much wider range than any individual study, finding no interactions between them. Third, to disentangle the effect of experiment length from those of other variables on egg production rates and egg hatching. This could otherwise create confusion when interpreting results of individual studies separately.

The results for the effect of copepod population exposure provided support for the existence of the acclimation/adaptation mechanism in the chronically exposed populations, first proposed by Colin and Dam (2002a), across populations of copepods distributed over a wide geographical range. The existence of this mechanism is revealed by significantly lower ingestion rates of toxic *Alexandrium* for the naïve populations, but also lower total ingestion rates and ingestion rates of non-toxic species (Table 1, Fig. 4). The clearly lower ingestion rates of *Alexandrium* denote the lower performance of the naïve populations with this toxic dinoflagellate. Lower total ingestion rates, also with sole diets of non-toxic food, were shown using mixed diet experiments by Colin and Dam (2002a). This overall lower biomass ingestion has the consequence of significantly lower overall egg production rates (Fig. 2). Thus lower egg production would be mainly a secondary effect of lower ingestion of biomass. In fact, several previous works have demonstrated

that naïve populations have lower ingestion and respiration rates than chronically exposed ones (Colin and Dam, 2003) hence they would be suffering physiological impairment due to starvation. Avery et al. (2008) and Finiguerra et al. (2013) suggested that the fitness drop of naïve populations is consistent with the effect of starvation, therefore the adaptation mechanism copes with starvation.

For egg hatching success, the effect of chronic exposure was overall nearly significant, and it was significant above the threshold for the negative effect of toxin, showing significantly lower egg hatching success for the naïve populations (see Results).

Overall, the negative effect of *Alexandrium* on physiology and reproduction of *Acartia* is only clear for egg hatching (above the threshold) and mortality. In the ingestion and egg production rates, which are directly related, it is only apparent when comparing naïve with chronically exposed populations.

The effect of other biotoxins and pollutants was also tested in marine planktonic copepods. Egg production rates and egg hatching success are heavily affected in a dose-dependent manner by a variety of pollutants (heavy metals, organic compounds) (Hussain et al. 2020). Other phytoplankton toxins, such as diatom oxylipins, have a strong effect in egg hatching and sometimes egg production rates, but no effect on ingestion rates (Ruocco et al. 2020). Other phytoplankton toxins, such as domoic acid, were never found to have a toxic effect on copepods (Maneiro et al. 2005).

The role of adaptive evolution in the interaction between these two planktonic organisms was claimed by Dam and collaborators in several works using *Acartia hudsonica* populations from the Gulf of Maine (Colin and Dam, 2002a and b, 2003, 2004, S.P. 2007; Avery and Dam, 2007; Zheng et al., 2011, Finiguerra et al., 2014). Their experiments employed both “natural” resistant wild populations and “induced” resistant

populations through generations of laboratory cultures. The proximal physiological mechanism responsible for this tolerance remains unknown. A mutation in the sodium channel which affects its intracellular inactivation gate was initially found to be associated with resistant populations (Chen et al., 2010; Finiguerra et al., 2013; Chen et al., 2015). However, further analysis showed that this mutation does not provide an adaptive benefit (Finiguerra et al., 2014, 2015). Other possible explanations for the mechanism are that adapted populations have a lower toxin absorption efficiency than naïve populations (Dam and Haley, 2011). Roncalli et al. (2017) showed that *Calanus* regulated genes encoding digestive enzymes, possibly to complement channel resistance by limiting toxin assimilation via the digestive process. This would constitute a gut detoxifying mechanism.

The limitation of our approach to study copepod adaptation is that we can only record presence or absence of chronic exposure of populations for most of our data sets. While chronic exposure is a requirement for the presence of adaptation, this needs to be demonstrated with common-garden designs or transgenerational designs that also remove maternal effects and account for genotypic adaptation only. However, a big portion of our data includes demonstration of adaptation, whether only genotypic or including maternal effects. Specifically, adaptation was tested in 75 % of the data employed for the analysis of total ingestion rates, 80 % of the data for the analysis of egg production rates and 77 % of the data for egg hatching. Table S1 shows, for each of the studies, whether adaptation was tested and how.

During the last decades, in the field of evolutionary ecology, several experimental works have suggested the existence of relatively fast mechanisms of adaptation in planktonic systems. There are examples in phytoplankton, with a single species or two interacting species (Collins and Bell, 2006; Hiltunen et al., 2014) and also in copepods, showing adaptation to warming and acidification (Dam et al. 2021; Brennan et al. 2022; Sasaki and Dam, 2021; de Juan et al. 2023a, 2023b). However, the importance of the *Acartia-Alexandrium* interaction relies in the fact that it would be the only mechanism of adaptive evolution demonstrated to work in the natural environment, in a planktonic biotic interaction, specifically, a grazer-producer interaction.

CRediT authorship contribution statement

Aldo Barreiro Felpeto: Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Máximo Frangópulos Rivera:** Writing – review & editing, Supervision, Data curation, Conceptualization. **Vitor Manuel Vasconcelos:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The present work was supported by contracts UIDB/04423/2020 and UIDP/04423/2020 from FCT (Portugal) to ABF and by grant MEC80190094 from CONICYT- ANID (Chile) to MFR and ABF. MFR also thanks ANID – Millennium Science Initiative Program (ICN2021-002) and ANID BASAL (FB210018), Cape Horn International Center (CHIC), Universidad de Magallanes. Finally, ABF and MFR also thanks ANID-Vinculación Internacional FOVI220159 and Subsidio interno para

Programas de Investigación en la Universidad de Magallanes (2022), Proyecto IMGLOTOX.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2024.102659.

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