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PROCEEDINGS B

# Cell-growth gene expression reveals a direct fitness cost of grazer-induced toxin production in red tide dinoflagellate prey

Gihong Park and Hans G. Dam

**Article citation details**
*Proc. R. Soc. B* **288**: 20202480.
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**Review timeline**

| | |
|---|---|
| Original submission: | 24 September 2020 |
| 1st revised submission: | 5 October 2020 |
| 2nd revised submission: | 21 December 2020 |
| Final acceptance: | 18 January 2021 |

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

# Review History

## Decision letter (RSPB-2020-2356.R0)

28-Sep-2020

Dear Dr Dam:

Thank you for submitting your manuscript RSPB-2020-2356 entitled "Cell-Growth Gene Expression Reveals Direct Fitness Cost of Grazer-Induced Toxin Production in Red Tide Dinoflagellate Prey" to Proceedings B.

All manuscripts are assessed by a specialist member of the Editorial Board, who decides whether the manuscript is suitable for Proceedings B.

Competition for space is currently extremely severe and we receive many more good manuscripts than we are able to publish, and we give preference to those that present significant advances of broad interest. Unfortunately, your manuscript has been rejected at this stage, as it was considered to be too specialised for Proceedings B, and would not appeal to the majority of readers.

Please find below the specialist Board member's comments, which I hope you may find useful should you wish to submit your manuscript elsewhere.

Sincerely,
The Proceedings B Team
mailto:proceedingsb@royalsociety.org

Board Member
Comments to Author(s):
This appears to be a well conducted study with important findings: there is little quantitative data on costs of induced defences and phenotypic plasticity in general, so the results here could be a very valuable addition to scientific literature. However, all this said, the manuscript comes across as if were written for specialist audience and therefore, it might not have the broad conceptual appeal as typically expected from Proc B papers. Hence, it might be a better fit to more specialised journal.

# RSPB-2020-2480.R0

## Review form: Reviewer 1

**Recommendation**
Accept with minor revision (please list in comments)

**Scientific importance: Is the manuscript an original and important contribution to its field?**
Excellent

**General interest: Is the paper of sufficient general interest?**
Good

**Quality of the paper: Is the overall quality of the paper suitable?**
Good

**Is the length of the paper justified?**
Yes

**Should the paper be seen by a specialist statistical reviewer?**
No

**Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.**
No

**It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.**

**Is it accessible?**
No

**Is it clear?**
N/A

**Is it adequate?**
N/A

**Do you have any ethical concerns with this paper?**
No

**Comments to the Author**
The study presents a novel approach to measure fitness costs in inducible defense systems, which is presented here using the predator-prey interaction of a harmful dinoflagellate and its copepod grazers. The presented study integrates gene expression analysis based on RT-qPCR into an ecologically relevant research question and thus demonstrates the knowledge gain that can be achieved by combining approaches, i.e. by integrating functional genomics into ecology. The study further develops and implements the relevant equations to calculate the potential costs based on the measured parameters and thus allows to estimate the inducible defense costs separately from the grazing rates. This in turn allows to correct the grazing rates due to the reduced growth rates caused by the induced defense system, which were not considered before. This approach is therefore a novelty and can be used by many others working in this field, and is worthy of publication. However, I think that some cross-validations are missing, but they do not question the concept and the study, so they could potentially be done in follow-up work but should be mentioned in the discussion. The idea and the fact that the method presented works are still valid and should therefore be made available to other scientists. I have some open questions which should be clarified before publication and which will help me to finally evaluate the work done. These questions as well as further suggestions and comments are described in detail below.

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Line 180: Please write the full name of two genes once.

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Line 208: Fitness Costs: Have you considered some kind of control treatment to assess fitness costs, i.e. in nutrient-limited growth conditions where growth rates have been reduced. Thus, the ratio of Cyc(treatment/control) can be correlated with reduced growth rates without possibly confounding it with grazing rates. Or why do you think that such a control is not necessary to evaluate or validate your results? Considerations regarding this should be included in the discussion for follow-up studies or as information to other scientists who are interested in this method.

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Line 240 / 241: Again, it is confusing when you talk about control bottles and then find that there was no interaction between grazer and time (although controls do not have copepods)

Line 243: grazer --> treatment, see comments above

Line 244: Refers to growth rates in general: Growth rates in both control and treatment are negative at the beginning of the experiment, indicating losses (or temporary cysts?) at the

beginning of the experiment, probably due to the experimental procedure. I therefore think it is worth considering the first 24h as an acclimatization phase and to use only the values from day 1 to day 4 for all calculations, because the first counts might lead to a skewed pattern. For example, the fit in Figure 3A would probably also get a better r2 if the amount of negative growth rates would be reduced. However, this procedure would affect many results and I wonder how big the effect would be. Have you thought about or discussed this option? And if so, what reason do you have to keep the initial time points?

Line 257: Do you mean with higher stability a more stable expression? Can you show the expression of the reference genes in the appendix?

Line 273-275: I do not really understand this, it probably needs one sentence more to be explained. Did you calculate the growth rate for grazer treatment here based on the cyc gene expression in the control?

Line 300: 'unequivocal demonstration and quantification' is a bit exaggerated. Perhaps a 'novel approach' would be better. Some things can still be questioned (e.g. see comment on further controls above).

Line 304: Based on what estimate will fitness costs be balanced by an 'equivalent' reduction in grazing mortality?

Line 309-311: To which figure are you referring here with this statement?

Line 319: Figure 2D does not exist.

Line 340-345: For both sentences, add the respective figures to which the statement refers.

Line 366: should read 'decrease'

## Review form: Reviewer 2

**Recommendation**
Accept with minor revision (please list in comments)

**Scientific importance: Is the manuscript an original and important contribution to its field?**
Good

**General interest: Is the paper of sufficient general interest?**
Good

**Quality of the paper: Is the overall quality of the paper suitable?**
Good

**Is the length of the paper justified?**
Yes

**Should the paper be seen by a specialist statistical reviewer?**
No

**Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.**
No

**It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.**

**Is it accessible?**
N/A

**Is it clear?**
Yes

**Is it adequate?**
Yes

**Do you have any ethical concerns with this paper?**
No

**Comments to the Author**
Referee comments to the manuscript RSPB-2020-2480; Cell-Growth Gene Expression Reveals Direct Fitness Cost of Grazer-Induced Toxin Production in Red Tide Dinoflagellate Prey
Authored by G. Park, and H. Dam

The authors present a novel take to a timely and unresolved enigma; why are there no measurable costs associated with toxin production in harmful algae? Harmful algae produce up to ten times more toxins in the presence of grazers, yet the induced cells appear to have the same fitness as less toxic cells. Ecological theory predicts a cost, but experimental data does not support it. Here the authors utilize genetic markers correlated to growth rate, to be able to estimate growth rate in treatments where the alga are exposed to direct grazing (to disentangle costs from grazing mortality). The authors show that the expression of the cyclin gene of choice is indeed reduced in grazed cultures. The authors use this to calculate the cost and estimate the corresponding reduction in growth rate to be 32 %. This is an exciting finding, partly because it may provide a first lead to the illusive costs of toxin production in harmful algae, but also because it may provide a new lead to the mechanism leading to increased toxicity in grazer induced harmful algae. The study is well performed and the manuscript well written, I only have one major concern regarding the design and interpretation of the experiment:

That cyclins are correlated to growth rate in control cultures does not mean that the same relationship is necessarily valid for the grazed cultures. A large proportion of genes may be differentially regulated in algae exposed to grazers (Amato et al 2018, ISME journal, 12(6), 1594-1604.), and if cyclins are too the relationship between cyclins and growth rate do not necessarily hold the way proposed here. This could relatively easily be tested as toxin production can be induced without direct grazing either by separating the cells from the grazers by a semipermeable mesh, or by adding the now known chemical cues from copepods that induce toxin formation (Selander et al 2015 PNAS 112(20) 6395-6400). When these strategies have been used in previous literature no direct fitness costs have resulted, which is yet a reason to test the validity of the direct coupling between cyclins and growth rate in grazer induced cultures.

Detailed comments:
Line 42 references on inducible defences in plankton should also include recent dinoflagellate references Prevett et al 2019, and Selander et al 2019
Prevett, A., Lindström, J., Xu, J., Karlson, B., & Selander, E. (2019). Grazer-induced bioluminescence gives dinoflagellates a competitive edge. Current Biology, 29(12), R564-R565.

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Line 64 The cues that induce toxin production in Alexandrium are now known : Selander et al 2015:
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Line 119-120 Misleading 20 Acartia per 500 ml does not correspond to three to four magnitudes lower consumer/prey rations than natural, it is a naturally occurring, but high concentration of grazers.
158: Eq 3 was I believe first used in this context by Guisande 2002, if inspired by that paper, maybe cite it:
Guisande, C., Frangopulos, M., Maneiro, I., Vergara, A. R., & Riveiro, I. (2002). Ecological advantages of toxin production by the dinoflagellate Alexandrium minutum under phosphorus limitation. Marine Ecology-Progress Series, 225, 169-176.

200-202 This is an assumption that i find is not valid as cyclins are indeed correlated to growth rate, but we do not know that it is unaffected by other things e.g. grazer induced toxin production. To hold it needs to be tested by inducing toxin production without grazing (by cues or caged grazers) and show that a comparable reduction in growth rate occur.

300 The statement first unequivocal demonstration and quantification of a direct fitness cost does not hold. The cyclins are a proxy for growth rate but the link to direct fitness cost is not proven here. A large proportion of genes may be altered in response to grazer presence, and it is thus possible that the cyclins are changed without corresponding change in growth rate.
A recent preprint e.g. show now fitness costs in another Alexandrium species held at various nutrient conditions and induced to higher toxicity by grazer cues, but the induced cells grow smaller. Depending of the role of the housekeeping genes used to normalize the gene expression data that type of effects may confound the result.
Ryderheim, F., Selander, E., & Kiørboe, T. (2020). Costs and benefits of toxin production in a dinoflagellate. bioRxiv.
304-305 the corresponding decrease in grazing rate is not shown in the results? Based on the cell counts it looks like grazing (+ cost) is equal to growth rate throughout exp? Also, I wonder how the copepods did in the experiment, mortality?, If mortality that will also lead to decreased grazing mortality.

611 toxin production rate denoted Rtox in materials and methods but μtox here, better to stick to one
Figure 3B is this growth rate over the whole period? And how can 20 out of 24 values be below zero when all cultures do have a net positive growth rate??

A thought came to mind: some cyclins moderate the transitions between different phases of the cell cycle, and toxins are known to mainly form in the G1 part of the cell cycle. I don't now about the one you monitored but if it could lead to an elongation of the G1 phase that may contribute to increased toxicity:
Taroncher-Oldenburg, G., Kulis, D. M., & Anderson, D. M. (1999). Coupling of saxitoxin biosynthesis to the G(1) phase of the cell cycle in the dinoflagellate Alexandrin fundyense: Temperature and nutrient effects. Natural Toxins, 7(5), 207-219.

Finally, dinoflagellates do induce both morphological changes (break up of chains) and bioluminescense in response to copepod grazers. Given that A catenella is a bioluminescent chain former it is possible that the cost estimate is not only related to toxin formation but could also be caused by additional and even multiple grazer induced responses.

Thank you for a well written and very interesting paper and good luck with the revision!

## Review form: Reviewer 3

**Recommendation**
Reject – article is not of sufficient interest (we will consider a transfer to another journal)

**Scientific importance: Is the manuscript an original and important contribution to its field?**
Good

**General interest: Is the paper of sufficient general interest?**
Acceptable

**Quality of the paper: Is the overall quality of the paper suitable?**
Good

**Is the length of the paper justified?**
Yes

**Should the paper be seen by a specialist statistical reviewer?**
No

**Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.**
No

**It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.**

**Is it accessible?**
Yes

**Is it clear?**
Yes

**Is it adequate?**
Yes

**Do you have any ethical concerns with this paper?**
No

**Comments to the Author**
"Cell growth gene expression reveals direct fitness costs of grazer induced toxin production in red tide dinoflagellate prey"

The authors present a new way to calculate costs of inducible defense in phytoplankton (toxin production) by using relative gene expression of a mitotic cyclin (cyc) gene. The manuscript is well written and focused. It is definitely of interest for plankton ecologist and is able to motivate more research about new methods determining costs of defenses.

I have a few comments:

-Separating fitness costs of inducible defenses: Such costs are often measured by exposing prey to predators without allowing mortality by grazing. As the defense is inducible, predator related cues may be responsible to induce defenses and no direct contact with predators is necessary. That allows to estimate fitness costs directly by measuring fitness relevant traits such as growth and reproduction without having direct mortality involved. The authors argue that direct predation may result in stronger defense development. However, according to the literature (cited by the authors) supporting this argument a defense in form of toxin production was also measurable by exposing algae just to cues alone. To my opinion a combination of both approaches could have some benefits. In the predator cue treatments one could measure fitness costs of toxin production by directly following fitness relevant parameters (growth) without confounding mortality by grazing. Comparing then toxin production in treatments with only predator cues and treatments with cues and predators would allow to measure differences in toxin production and thereby estimating costs to toxin production also in treatments including mortality by predators.

-Estimates from a single gene (cyc) to refer to growth and calculate growth rate reduction and fitness costs. Would this mean that the expression of the gene is mainly linked to somatic growth, but not influenced by any other fitness related parameters?

-Toxin production and cyc expression: From figure 2 it looks like that not only the gene expression is different between control and toxin producing phenotypes, but that also the variation around the mean is different (Fig. 2C). Standard deviations are much larger in controls than in treatments. Is this just an effect from less variable growth rates in toxin producing algae? It is difficult to estimate this point from Fig 3 C. Could this otherwise also mean that there is an interaction between toxin production and cyc expression per unit growth? In such a case it would not allow to measure fitness costs by using the expression of cyc, or?

-To my opinion one way to control for such an interaction would be to measure cyc gene expression at different growth rates (f.e. along a resource gradient) with and without toxin production and compare the slopes of the regression as seen in Fig. 3A but for both, control and toxin producing phenotypes. Using only the slope of Fig 3A of control animals for cost calculations would mean that the relationship between cyc overexpression based on the reference gene and growth has to be identical between control and toxin producing phenotypes. Is there enough evidence to be sure about that?

## Decision letter (RSPB-2020-2480.R0)

30-Nov-2020

Dear Dr Dam:

Your manuscript has now been peer reviewed and the reviews have been assessed by an Associate Editor. The reviewers' comments (not including confidential comments to the Editor) and the comments from the Associate Editor are included at the end of this email for your reference. As you will see, the reviewers and the Associate Editor have raised some issues with your manuscript and we would like to invite you to revise your manuscript to address them.

We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Associate Editor, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available we may invite new reviewers. Please note that we cannot guarantee eventual acceptance of your manuscript at this stage.

To submit your revision please log into http://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions", click on "Create a Revision". Your manuscript number has been appended to denote a revision.

When submitting your revision please upload a file under "Response to Referees" in the "File Upload" section. This should document, point by point, how you have responded to the reviewers' and Editors' comments, and the adjustments you have made to the manuscript. We require a copy of the manuscript with revisions made since the previous version marked as 'tracked changes' to be included in the 'response to referees' document.

Your main manuscript should be submitted as a text file (doc, txt, rtf or tex), not a PDF. Your figures should be submitted as separate files and not included within the main manuscript file.

When revising your manuscript you should also ensure that it adheres to our editorial policies (https://royalsociety.org/journals/ethics-policies/). You should pay particular attention to the following:

Research ethics:
If your study contains research on humans please ensure that you detail in the methods section whether you obtained ethical approval from your local research ethics committee and gained informed consent to participate from each of the participants.

Use of animals and field studies:
If your study uses animals please include details in the methods section of any approval and licences given to carry out the study and include full details of how animal welfare standards were ensured. Field studies should be conducted in accordance with local legislation; please include details of the appropriate permission and licences that you obtained to carry out the field work.

Data accessibility and data citation:
It is a condition of publication that you make available the data and research materials supporting the results in the article (https://royalsociety.org/journals/authors/author-guidelines/#data). Datasets should be deposited in an appropriate publicly available repository and details of the associated accession number, link or DOI to the datasets must be included in the Data Accessibility section of the article (https://royalsociety.org/journals/ethics-policies/data-sharing-mining/). Reference(s) to datasets should also be included in the reference list of the article with DOIs (where available).

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should also be fully cited and listed in the references.

If you wish to submit your data to Dryad (http://datadryad.org/) and have not already done so you can submit your data via this link
http://datadryad.org/submit?journalID=RSPB&manu=(Document not available), which will take you to your unique entry in the Dryad repository.

If you have already submitted your data to dryad you can make any necessary revisions to your dataset by following the above link.

For more information please see our open data policy http://royalsocietypublishing.org/data-sharing.

Electronic supplementary material:

All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI. Please try to submit all supplementary material as a single file.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rspb.[paper ID in form xxxx.xxxx e.g. 10.1098/rspb.2016.0049].

Please submit a copy of your revised paper within three weeks. If we do not hear from you within this time your manuscript will be rejected. If you are unable to meet this deadline please let us know as soon as possible, as we may be able to grant a short extension.

Thank you for submitting your manuscript to Proceedings B; we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Best wishes,
Professor Hans Heesterbeek
mailto: proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
Dear Authors

We have obtained three expert reviews on your submission, and they are all fairly positive albeit one the reviewers is in opinion that your paper is not of sufficient interest to Proc B. This is a matter of opinion (and this was my initial opinion too as you surely remember). However, since the critical reviewer does not raise any severe criticism towards your science, and the other two experts praise your work, I think it is fair to me to admit that my initial opinion might have been wrong. In particular, reading the excellently phrased report of the reviewer #2, I think I see the light now - her/his report phrases the conceptual problem in the way that even non-expert can see why your work is interesting and matters. Since all the referees made an array of suggestions how the manuscript could be still improved, I believe the manuscript requires still a substantial revision to be acceptable for publication. The detailed referee comments should be helpful in this respect, and I would think that referee #2's wording as to why the problem you are tackling is interesting would be worth thinking when revising the ms.

Best wishes
Juha Merilä

Reviewer(s)' Comments to Author:
Referee: 1
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Referee: 2
Comments to the Author(s).
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Authored by G. Park, and H. Dam

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Detailed comments:
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200-202 This is an assumption that i find is not valid as cyclins are indeed correlated to growth rate, but we do not know that it is unaffected by other things e.g. grazer induced toxin production. To hold it needs to be tested by inducing toxin production without grazing (by cues or caged grazers) and show that a comparable reduction in growth rate occur.

300 The statement first unequivocal demonstration and quantification of a direct fitness cost does not hold. The cyclins are a proxy for growth rate but the link to direct fitness cost is not proven here. A large proportion of genes may be altered in response to grazer presence, and it is thus possible that the cyclins are changed without corresponding change in growth rate.
A recent preprint e.g. show now fitness costs in another Alexandrium species held at various nutrient conditions and induced to higher toxicity by grazer cues, but the induced cells grow smaller. Depending of the role of the housekeeping genes used to normalize the gene expression data that type of effects may confound the result.
Ryderheim, F., Selander, E., & Kiørboe, T. (2020). Costs and benefits of toxin production in a dinoflagellate. bioRxiv.
304-305 the corresponding decrease in grazing rate is not shown in the results? Based on the cell counts it looks like grazing (+ cost) is equal to growth rate throughout exp? Also, I wonder how the copepods did in the experiment, mortality?, If mortality that will also lead to decreased grazing mortality.

611 toxin production rate denoted Rtox in materials and methods but µtox here, better to stick to one
Figure 3B is this growth rate over the whole period? And how can 20 out of 24 values be below zero when all cultures do have a net positive growth rate??

A thought came to mind: some cyclins moderate the transitions between different phases of the cell cycle, and toxins are known to mainly form in the G1 part of the cell cycle. I don't now about the one you monitored but if it could lead to an elongation of the G1 phase that may contribute to increased toxicity:
Taroncher-Oldenburg, G., Kulis, D. M., & Anderson, D. M. (1999). Coupling of saxitoxin biosynthesis to the G(1) phase of the cell cycle in the dinoflagellate Alexandrin fundyense: Temperature and nutrient effects. Natural Toxins, 7(5), 207-219.

Finally, dinoflagellates do induce both morphological changes (break up of chains) and bioluminescense in response to copepod grazers. Given that A catenella is a bioluminescent chain former it is possible that the cost estimate is not only related to toxin formation but could also be caused by additional and even multiple grazer induced responses.

Thank you for a well written and very interesting paper and good luck with the revision!

Referee: 3
Comments to the Author(s).
"Cell growth gene expression reveals direct fitness costs of grazer induced toxin production in red tide dinoflagellate prey"

The authors present a new way to calculate costs of inducible defense in phytoplankton (toxin production) by using relative gene expression of a mitotic cyclin (cyc) gene. The manuscript is well written and focused. It is definitely of interest for plankton ecologist and is able to motivate more research about new methods determining costs of defenses.

I have a few comments:

-Separating fitness costs of inducible defenses: Such costs are often measured by exposing prey to predators without allowing mortality by grazing. As the defense is inducible, predator related cues may be responsible to induce defenses and no direct contact with predators is necessary. That allows to estimate fitness costs directly by measuring fitness relevant traits such as growth and reproduction without having direct mortality involved. The authors argue that direct predation may result in stronger defense development. However, according to the literature (cited by the authors) supporting this argument a defense in form of toxin production was also measurable by exposing algae just to cues alone. To my opinion a combination of both approaches could have some benefits. In the predator cue treatments one could measure fitness costs of toxin production by directly following fitness relevant parameters (growth) without confounding mortality by grazing. Comparing then toxin production in treatments with only predator cues and treatments with cues and predators would allow to measure differences in toxin production and thereby estimating costs to toxin production also in treatments including mortality by predators.

-Estimates from a single gene (cyc) to refer to growth and calculate growth rate reduction and fitness costs. Would this mean that the expression of the gene is mainly linked to somatic growth, but not influenced by any other fitness related parameters?

-Toxin production and cyc expression: From figure 2 it looks like that not only the gene expression is different between control and toxin producing phenotypes, but that also the variation around the mean is different (Fig. 2C). Standard deviations are much larger in controls than in treatments. Is this just an effect from less variable growth rates in toxin producing algae? It is difficult to estimate this point from Fig 3 C. Could this otherwise also mean that there is an interaction between toxin production and cyc expression per unit growth? In such a case it would not allow to measure fitness costs by using the expression of cyc, or?

-To my opinion one way to control for such an interaction would be to measure cyc gene expression at different growth rates (f.e. along a resource gradient) with and without toxin production and compare the slopes of the regression as seen in Fig. 3A but for both, control and toxin producing phenotypes. Using only the slope of Fig 3A of control animals for cost calculations would mean that the relationship between cyc overexpression based on the reference gene and growth has to be identical between control and toxin producing phenotypes. Is there enough evidence to be sure about that?

## Author's Response to Decision Letter for (RSPB-2020-2480.R0)

See Appendix A.

# RSPB-2020-2480.R1 (Revision)

## Review form: Reviewer 1

**Recommendation**
Accept as is

**Scientific importance: Is the manuscript an original and important contribution to its field?**
Good

**General interest: Is the paper of sufficient general interest?**
Good

**Quality of the paper: Is the overall quality of the paper suitable?**
Excellent

**Is the length of the paper justified?**
Yes

**Should the paper be seen by a specialist statistical reviewer?**
No

**Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.**
No

**It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.**

**Is it accessible?**
Yes

**Is it clear?**
Yes

**Is it adequate?**
Yes

**Do you have any ethical concerns with this paper?**
No

**Comments to the Author**
The authors did a great job to respond to my critical points. Major points that have been improved are changes in the introduction to make the manuscript more accessible to a broader, more general readership (I had concerns about this); a clarification of methods and calculation of costs ( I was misled by the previous version) and a discussion part including suggestions how results could be validated in more detail by future experiments.

# Decision letter (RSPB-2020-2480.R1)

18-Jan-2021

Dear Dr Dam

I am pleased to inform you that your manuscript entitled "Cell-Growth Gene Expression Reveals a Direct Fitness Cost of Grazer-Induced Toxin Production in Red Tide Dinoflagellate Prey" has been accepted for publication in Proceedings B.

You can expect to receive a proof of your article from our Production office in due course, please check your spam filter if you do not receive it. PLEASE NOTE: you will be given the exact page length of your paper which may be different from the estimation from Editorial and you may be asked to reduce your paper if it goes over the 10 page limit.

If you are likely to be away from e-mail contact please let us know. Due to rapid publication and an extremely tight schedule, if comments are not received, we may publish the paper as it stands.

If you have any queries regarding the production of your final article or the publication date please contact procb_proofs@royalsociety.org

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Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

Thank you for your fine contribution. On behalf of the Editors of the Proceedings B, we look forward to your continued contributions to the Journal.

Sincerely,
Professor Hans Heesterbeek
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor:
Board Member: 1
Comments to Author:
Dear Authors

Thank you for revising your ms along the lines suggested by the reviewers. Both me and the referee who has read the revised version are happy with these revisions - well done.

Best wishes
Juha Merilä

Board Member: 2
Comments to Author:
The authors did a great job to respond to my critical points. Major points that have been improved are changes in the introduction to make the manuscript more accessible to a broader, more general readership (I had concerns about this); a clarification of methods and calculation of costs ( I was misled by the previous version) and a discussion part including suggestions how results could be validated in more detail by future experiments.

# Appendix A

**Park and Dam**
**Response to Editor and Reviewers:**
The reviewers' comments are in black color. Our responses are in red. Reference to changes in the manuscript refer to the clean copy. We attach an addendum that deals with two points: 1) Use of cage experiments (to mimic grazer cues) to demonstrate fitness cost of defense, and 2) Evidence that upregulation of STX gene and downregulation of the cyclin B gene depends on grazing pressure in the strain in our study, but is independent of grazing pressure in noninducible strains. Both points are relevant to issues brought by the reviewers. To this response we attach the track-changes edited version of the manuscript, so that it is clear where we made changes to the manuscript, and the supplementary information.

**Editor:**
We have obtained three expert reviews on your submission, and they are all fairly positive albeit one the reviewers is in opinion that your paper is not of sufficient interest to Proc B. This is a matter of opinion (and this was my initial opinion too as you surely remember). However, since the critical reviewer does not raise any severe criticism towards your science, and the other two experts praise your work, I think it is fair to me to admit that my initial opinion might have been wrong. In particular, reading the excellently phrased report of the reviewer #2, I think I see the light now - her/his report phrases the conceptual problem in the way that even non-expert can see why your work is interesting and matters. Since all the referees made an array of suggestions how the manuscript could be still improved, I believe the manuscript requires still a substantial revision to be acceptable for publication. The detailed referee comments should be helpful in this respect, and I would think that referee #2's wording as to why the problem you are tackling is interesting would be worth thinking when revising the ms.

Thank you for the positive feedback to our manuscript and your suggestions. Below are the detailed answers to all three reviewers. We have rewritten the abstract and parts of the introduction to make the paper more accessible to general reader. In response to comments and suggestions from the reviewers, we expanded the discussion, which now considers potential biases of the approach and suggests some experiments for cross validation. The title is slightly changed now to indicate that we reveal a cost of toxin production. Given that our manuscript was already at the maximum for length, we had to cut 14 references to balance the added text. None of these references were essential. We hope you will find the revised version of the manuscript and our responses to the reviewers acceptable.

**Reviewer 1:**

Comments to the Author(s).
The study presents a novel approach to measure fitness costs in inducible defense systems, which is presented here using the predator-prey interaction of a harmful dinoflagellate and its copepod grazers. The presented study integrates gene expression analysis based on RT-qPCR into an ecologically relevant research question and thus demonstrates the knowledge gain that can be achieved by combining approaches, i.e. by integrating functional genomics into ecology. The study further develops and implements the relevant equations to calculate the potential costs based on the measured parameters and thus allows to estimate the inducible defense costs separately from the grazing rates. This in turn allows to correct the grazing rates due to the reduced growth rates caused by the induced defense system, which were not considered before.
This approach is therefore a novelty and can be used by many others working in this field, and is worthy of publication. However, I think that some cross-validations are missing, but they do not question the concept and the study, so they could potentially be done in follow-up work but should be mentioned in the discussion. The idea and the fact that the method presented works are still valid and should therefore be made available to other scientists. I have some open questions which should be clarified before

publication and which will help me to finally evaluate the work done. These questions as well as further suggestions and comments are described in detail below.

We thank the reviewer for their support of the manuscript. We have added to the discussion to suggest ways to cross-validate the approach (see lines 344-363 of the revised manuscript).

Line 46: A matter of taste, but I suggest to delete 'dramatic' here (and also in line 115, 293, and 306).
The word “dramatic” has been deleted accordingly.

Line 179: Please add how the primer efficiency was estimated (e.g. by standard curves).
“Relative gene expression (RGE) levels of the target genes were compared to those in the reference gene according to Pfaffl method corrected by standard curves to estimate the primer efficiency [49]”.

Line 180: Please write the full name of two genes once.
Line now reads: “The reference genes, *lbp* (luciferin-binding protein) and *cob* (cytochrome b), were used to normalize the gene expression level [41,50]”.

Line 186/187 and Fig. 1C: Here it says that the µnet refers to the growth rate in the copepod treatment, but Fig. 1C obviously shows the net growth rate also for the control. Therefore, it may be better to label the axis only with "growth rate" to not confuse the readers with equation 4. Another possibility would be to display both "traditional" growth rates and newly estimated growth rates based on the methods presented here in the same figure (but then move it to Figure 4). Also, for completeness, Figure 1 should include a figure showing the daily toxin production rates.
Thank you. Good suggestion. The figure legend now reads:
“Cell concentration (A), cell toxin content (B), cell growth rate versus time (C), and toxin production rate (µtox) versus cell growth rate (D) during the grazing assay. The growth rate in the copepod treatment refers to the net growth rate (µnet). Lines are regression fits”.

We also present a new figure of the growth rate versus toxin production rate in the supplementary materials (Appendix S3F). We do this to avoid overloading figure 1.

Line 208: Fitness Costs: Have you considered some kind of control treatment to assess fitness costs, i.e. in nutrient-limited growth conditions where growth rates have been reduced. Thus, the ratio of Cyc(treatment/control) can be correlated with reduced growth rates without possibly confounding it with grazing rates. Or why do you think that such a control is not necessary to evaluate or validate your results? Considerations regarding this should be included in the discussion for follow-up studies or as information to other scientists who are interested in this method.
We appreciate the reviewer’s suggestion, although it was unclear if the reviewer referred entirely to the control (no grazers). Indeed, a further validation of the approach would be to run assays under nutrient limitation, and see if the correlation of cell growth and RGE of *cyc* gene would hold. Fig. 3A of the manuscript shows RGE of *cyc* and cell division rate for the control. Notice that there is a wide range of growth rates, some of which may be what one finds under nutrient resource limitation.

We agree that further work is needed to validate our proposed approach. We are conducting follow-up experiments to test for light x nutrient x grazer as controlling factors, i.e. high (~600 µM $m^{-2}$ $s^{-1}$), low (~20 µM $m^{-2}$ $s^{-1}$), and no light intensity; Nutrient replete (F/2–si) and limited (FSW) conditions; and presence and absence of grazer. Thus, the question of the gene expression in the multi-resource variables will still be pursued. We briefly mention that costs assays should be run under resource replete and limited conditions in the discussion (lines 344-363).

Line 237: I suggest to replace “grazer" with "treatment" because the interaction refers to the

presence/absence of the grazer if I have understood it correctly. Control and treatment were not tested separately by you.
“Grazer" is replaced with "treatment”.

Line 240 / 241: Again, it is confusing when you talk about control bottles and then find that there was no interaction between grazer and time (although controls do not have copepods)
“Control bottles" is replaced with "controls”.
“Grazer" is replaced with "treatment”.

Line 243: grazer --> treatment, see comments above
“Grazer" is replaced with "treatment”.

Line 244: Refers to growth rates in general: Growth rates in both control and treatment are negative at the beginning of the experiment, indicating losses (or temporary cysts?) at the beginning of the experiment, probably due to the experimental procedure. I therefore think it is worth considering the first 24h as an acclimatization phase and to use only the values from day 1 to day 4 for all calculations, because the first counts might lead to a skewed pattern. For example, the fit in Figure 3A would probably also get a better r2 if the amount of negative growth rates would be reduced. However, this procedure would affect many results and I wonder how big the effect would be. Have you thought about or discussed this option? And if so, what reason do you have to keep the initial time points?
Yes, growth rates in both control and treatment are negative during the first 8 h of the experiment, but as expected they are lower in the grazer treatment. We did not observe cyst formation during the experiment in either controls or treatments. We prefer not to ignore the data before 24 h for several reasons. First, our comparisons are relative (treatment vs control). Second, the early toxin gene expression indicates that the prey response to the threat is quick. Third, including the first 24 h provides a conservative estimate of the fitness cost of defense (Appendix S4 Table in supplementary materials). Finally, removing the first 8 h of the experiment does not improve the statistical fit.

Line 257: Do you mean with higher stability a more stable expression? Can you show the expression of the reference genes in the appendix?
As the reviewer suggested, Appendix S1C in supplementary materials shows gene expression of references *lbp* and *cob* in the control and treatment. The expression level of *lbp* was more stable with the maximum fold-change of 1.77 in the control and 1.47 in the treatment, compared to that of cob (2.49-fold and 2.78-fold, respectively). Hence, the *lbp* gene was chosen to normalize the gene expression levels in the control (ANOVA, $p>0.07$) and treatment (ANOVA, $p>0.259$). A statement has been added to the methods to indicate this (lines 179-180).

Line 273-275: I do not really understand this, it probably needs one sentence more to be explained. Did you calculate the growth rate for grazer treatment here based on the cyc gene expression in the control?
It was a mistake for us to put regression lines in Figs 3A and 3B because this led reviewer 1 and 3 to think we used those regressions to calculate the defense fitness cost. In the revised version, we have deleted the regressions and instead show the scatter plots with the nonparametric correlation coefficient, Kendall’s Tau. The point of Fig. 3 was to show a significant and positive correlation between relative expression of cyclin gene and cell growth rate in the control (Fig. 3A). Such relationship suggests that a reduction in REG of *cyc* in the treatment translates to a reduction of the cell growth rate. Fig 3 B is to confirm the putative trade-off in Fig. 1D, which includes grazing losses by consumers, and was not for the evaluation of the defense fitness cost. The cost was derived from the proportion of relative expression of cyclin gene between cells in the control (constitutive toxin production) and the copepod treatment (induced toxin production) (Eq. 7).

The text has been changed to state: “Relative gene expression of *cyc* was positively correlated to cell growth rate in the control (Fig. 3A; $\tau$=0.46, $p$=0.002, $n$=24), which suggests that *cyc* is transcriptionally regulated with cell growth. Moreover, a trade-off was confirmed by the significantly negative correlation between toxin production rate measured by HPLC and RGE of *cyc* (Fig. 3B, $\tau$=-0.38, $p$=0.01, $n$=24). Thus, we used Eq. 7 to calculate the fitness cost incurred by grazer-induced defense.” (lines 268-272).

Line 300: ‘unequivocal demonstration and quantification’ is a bit exaggerated. Perhaps a 'novel approach' would be better. Some things can still be questioned (e.g. see comment on further controls above).
Suggestion accepted. “Unequivocal” is replaced with “novel.”

Line 304: Based on what estimate will fitness costs be balanced by an 'equivalent' reduction in grazing mortality?
“An equivalent reduction" is replaced with "a reduction of ”. That is, the calculated fitness loss of 32% in cell growth rate is nearly balanced by the estimated 29% reduction in grazing rate loss. (see appendix S4)

Line 309-311: To which figure are you referring here with this statement?
The text has been changed to state: “This inducible response may be advantageous because resources associated with a costly defense (toxin production) are used when needed, and otherwise allocated to other functions like growth and reproduction [1,23,24].” (lines 303-305)

Line 319: Figure 2D does not exist.
Our mistake. That mention of Fig. 2D referred to the information presented in the supplementary material (Appendix S3D). Error fixed in line 311.

Line 340-345: For both sentences, add the respective figures to which the statement refers.
Done. Line now reads: “In time-dependent experiments, both the actual toxin content measured by HPLC and the relative expression of STX genes were significantly increased in response to grazers, whereas both of these measurements remained unchanged in cells not exposed to grazers (Fig. 1B and 2A-B). Moreover, cell division rate calculated from the relative expression of the cell growth gene (*cyc*) was significantly lower in cells exposed to grazers than unexposed cells (Fig. 3B), confirming the trade-off between toxin production rate and cell division rate suggested by Fig. 1D.” (lines 332-337)

Line 366: should read ‘decrease’
“Decreased" is replaced with "decrease”.

**Reviewer 2:**

Comments to the Author(s).
Referee comments to the manuscript RSPB-2020-2480; Cell-Growth Gene Expression Reveals Direct Fitness Cost of Grazer-Induced Toxin Production in Red Tide Dinoflagellate Prey
Authored by G. Park, and H. Dam

The authors present a novel take to a timely and unresolved enigma; why are there no measurable costs associated with toxin production in harmful algae? Harmful algae produce up to ten times more toxins in the presence of grazers, yet the induced cells appear to have the same fitness as less toxic cells. Ecological theory predicts a cost, but experimental data does not support it. Here the authors utilize genetic markers correlated to growth rate, to be able to estimate growth rate in treatments where the alga are exposed to direct grazing (to disentangle costs from grazing mortality). The authors show that the expression of the cyclin gene of choice is indeed reduced in grazed cultures. The authors use this to calculate the cost and estimate the corresponding reduction in growth rate to be 32 %. This is an exciting finding, partly because

it may provide a first lead to the illusive costs of toxin production in harmful algae, but also because it may provide a new lead to the mechanism leading to increased toxicity in grazer induced harmful algae. The study is well performed and the manuscript well written, I only have one major concern regarding the design and interpretation of the experiment:

That cyclins are correlated to growth rate in control cultures does not mean that the same relationship is necessarily valid for the grazed cultures. A large proportion of genes may be differentially regulated in algae exposed to grazers (Amato et al 2018, ISME journal, 12(6), 1594-1604.), and if cyclins are too the relationship between cyclins and growth rate do not necessarily hold the way proposed here. This could relatively easily be tested as toxin production can be induced without direct grazing either by separating the cells from the grazers by a semipermeable mesh, or by adding the now known chemical cues from copepods that induce toxin formation (Selander et al 2015 PNAS 112(20) 6395-6400). When these strategies have been used in previous literature no direct fitness costs have resulted, which is yet a reason to test the validity of the direct coupling between cyclins and growth rate in grazer induced cultures.

We thank the reviewer for their enthusiastic support of our work. We also appreciate the reviewer's concern. We provide a detailed explanation of the rationale for our experiment (see point 1 in addendum) in which we forego to use the indirect exposure approach for the very reasons that the reviewer mentions—it is operationally difficult to establish a cost of defense using the grazer cues. Further, the grazer cues themselves may create the same differential gene expression issue the reviewer is concerned about.

As for the reviewer's concern, the crucial issue is whether the presence of grazers will differentially affect the cell division rate in ways other than by the cost of inducing toxin production. We argued this was unlikely the case. We state (as in the previous version)~~d~~ on lines 200-203: "*A significant and positive correlation between relative expression of cyclin gene (cyc) and cell growth rate in the absence of grazers (Fig. 3A), implies that we can use the former as a proxy of cell division rate during the experiment.* ***This assumes that grazing losses do not intrinsically affect the cell cycle, and thus the cell division rate as demonstrated earlier*** *[51]."* Ref. 51 (Chang and Dam 1993) is a study that shows that grazing by copepods does not intrinsically affect the cell division cycle, hence cell growth rate. Since we saw a decrease in RGE of the cell growth rate gene, *cyc*, in the presence of grazers in our experiments, we inferred that this represents a reduction of the cell division rate linked to toxin production. Point 2 of the addendum presents indirect evidence in support of this assumption. Namely, strains of *Alexandrium catenella* in which toxin production is not grazer-induced do not upregulate RGE of *stxA* or downregulate RGE of *cyc*. The paper by Amato et al (2018) shows that cyclin genes can be downregulated or upregulated for a diatom in the presence of grazers, but did not establish a direct correlation to growth rate. However, upregulating cyclin and STX gene expression simultaneously would fly in the face of predictions from trade-off theory—the cells would simultaneously increase toxin production and cell division. So, we think this is an unlikely scenario. By contrast, additional defenses that downregulate RGE of *cyc* would lead to an overestimation of the fitness cost of toxin production. In that case, Eq. 7 actually measures the cost of all defenses. This is now acknowledged on lines 344-363.

In retrospect, there may be another way to unequivocally test the validity of the approach— by measuring RGE of *cyc* in the presence and absence of grazers (as done in our study), and the frequency of cells in the different phases of the cell cycle and the phase durations in a diel cycle to independently calculate cell division rates [equation 3 in Ref. 51]. We make mention of this in the discussion now (lines 344-363).

Detailed comments:
Line 42 references on inducible defences in plankton should also include recent dinoflagellate references Prevett et al 2019, and Selander et al 2019
Prevett, A., Lindström, J., Xu, J., Karlson, B., & Selander, E. (2019). Grazer-induced bioluminescence

gives dinoflagellates a competitive edge. Current Biology, 29(12), R564-R565.
Selander, E., Berglund, E., Engström, P., Berggren, F., Eklund, J., Harðardóttir, S., . . . Andersson, M. (2019). Copepods drive large-scale trait-mediated effects in marine plankton. Science Advances, 5(2), eaat5096.
All three references have been added. Thanks

Line 64 The cues that induce toxin production in Alexandrium are now known : Selander et al 2015: Selander, E., Kubanek, J., Hamberg, M., Andersson, M. X., Cervin, G., & Pavia, H. (2015). Predator lipids induce paralytic shellfish toxins in bloom-forming algae. Proceedings of the National Academy of Sciences, 112(20), 6395-6400.
Reference added.

Line 119-120 Misleading 20 Acartia per 500 ml does not correspond to three to four magnitudes lower consumer/prey rations than natural, it is a naturally occurring, but high concentration of grazers.
Incubations used 500 ml poly-carbonate bottles containing approximately 250 cells $ml^{-1}$ of *Alexandrium catenella*, and 20 copepods, yielding **a grazer:prey** ratio of 0.00016. This ratio is orders of magnitude lower than found for **bloom conditions**, ruling out bias due to overabundance of grazers in the incubations. We now state bloom conditions instead of natural conditions in line 121.

158: Eq 3 was I believe first used in this context by Guisande 2002, if inspired by that paper, maybe cite it:
Guisande, C., Frangopulos, M., Maneiro, I., Vergara, A. R., & Riveiro, I. (2002). Ecological advantages of toxin production by the dinoflagellate Alexandrium minutum under phosphorus limitation. Marine Ecology-Progress Series, 225, 169-176.
Equation 3 is not needed and has been deleted.

200-202 This is an assumption that i find is not valid as cyclins are indeed correlated to growth rate, but we do not know that it is unaffected by other things e.g. grazer induced toxin production. To hold it needs to be tested by inducing toxin production without grazing (by cues or caged grazers) and show that a comparable reduction in growth rate occur.
See point 1 in addendum and reply to reviewer 1 about use of cages for detecting fitness cost of toxin production. We acknowledge that further work is required and this is added to the discussion (lines 344-363). See also response to last point of reviewer 3 (below).

300 The statement first unequivocal demonstration and quantification of a direct fitness cost does not hold. The cyclins are a proxy for growth rate but the link to direct fitness cost is not proven here. A large proportion of genes may be altered in response to grazer presence, and it is thus possible that the cyclins are changed without corresponding change in growth rate.
A recent preprint e.g. show now fitness costs in another Alexandrium species held at various nutrient conditions and induced to higher toxicity by grazer cues, but the induced cells grow smaller. Depending of the role of the housekeeping genes used to normalize the gene expression data that type of effects may confound the result.
Ryderheim, F., Selander, E., & Kiørboe, T. (2020). Costs and benefits of toxin production in a dinoflagellate. bioRxiv.
We rewrote this section. Instead of unequivocal, we now say we present a novel approach to calculate cost of defense. See also earlier replies about this issue (response to reviewer 1, their mention of line 257), and rewrite in the discussion of a possible further experiment to validate the approach. Regarding the housekeeping genes: Zhuang et al. (2013) also measured the reference genes (*lbp* and *gapdh*) over the 24-h cell cycle of *Alexandrium catenella*. Although the expression level of *gapdh* and *lbp* relative to total RNA amount fluctuated, the changes were not significant (ANOVA, $p > 0.7$). The expression level of *lbp* was more stable with the maximum fold-change of 2.18, compared to a 4.29-fold of *gapdh*.

Zhuang, Y., Zhang, H., & Lin, S. (2013). Cyclin B gene and its cell cycle-dependent differential expression in the toxic dinoflagellate *Alexandrium fundyense* Atama group I/Clade I. *Harmful Algae*, 26(0), 71-9.

304-305 the corresponding decrease in grazing rate is not shown in the results? Based on the cell counts it looks like grazing (+ cost) is equal to growth rate throughout exp? Also, I wonder how the copepods did in the experiment, mortality?, If mortality that will also lead to decreased grazing mortality.
The corresponding decrease in grazing rate was shown in the Appendix S4 in supplementary materials. The copepod survival (%) was over 92% in the control and treatment and there was no significant difference ($p$=0.92).

611 toxin production rate denoted Rtox in materials and methods but µtox here, better to stick to one
Figure 3B is this growth rate over the whole period? And how can 20 out of 24 values be below zero when all cultures do have a net positive growth rate??
In retrospect Eq. 3 is not necessary. So, we deleted it. The calculations all use µtox (Eq. 2). Figure 3B is over the whole period in control and treatment, and correctly refers to µtox. Because the growth rates in Fig. 3B are calculated by the regression (Fig. 3A) to confirm the trade-off, these values do not match with the cell growth rates in Fig. 1D. Plus, because two samples for PST content were analyzed out of four independent replicates per test, the values do not correspond to the cell growth rates in Fig. 1C. This was all spelled out in the methods section of the first version of the paper.

A thought came to mind: some cyclins moderate the transitions between different phases of the cell cycle, and toxins are known to mainly form in the G1 part of the cell cycle. I don't know about the one you monitored but if it could lead to an elongation of the G1 phase that may contribute to increased toxicity: Taroncher-Oldenburg, G., Kulis, D. M., & Anderson, D. M. (1999). Coupling of saxitoxin biosynthesis to the G(1) phase of the cell cycle in the dinoflagellate Alexandrin fundyense: Temperature and nutrient effects. Natural Toxins, 7(5), 207-219.
We appreciate the reviewer's thought. However, we would argue that the elongated G1 is the consequence instead of the cause of toxin production. Cells use the G1 phase to accumulate necessary resources and wait for the licensing signal (S phase cyclin) to enter the S phase. Because the production of toxin consumes extra resource, the licensing signal is delayed, and the G1 phase become longer as a result. This conjecture can be eventually tested by the follow up experiment we suggest in lines 344-363.

Finally, dinoflagellates do induce both morphological changes (break up of chains) and bioluminescense in response to copepod grazers. Given that A catenella is a bioluminescent chain former it is possible that the cost estimate is not only related to toxin formation but could also be caused by additional and even multiple grazer induced responses.
Thank you for the insightful comment. We know acknowledge in the discussion (lines 344-363) that **if there are multiple forms of defense that downregulate the *cyc* expression**, then our approach actually measures the cost of all forms of defense, and consequently overestimates the cost of toxin production. In recognition of the unresolved issues about our method, we made a subtle but important change to the title of the paper. It now indicates *a cost* instead of the cost of toxin production. Finally, the moderate strain in Fig. 3 of the addendum actually decreases its size in response to increasing grazing pressure and simultaneously increases its growth rate, yet this is independent of expression of the cyc gene. We are working on a separate manuscript that describes these experiments.

Thank you for a well written and very interesting paper and good luck with the revision!
We appreciate your constructive comments and insights!

**Reviewer 3:**

Comments to the Author(s).
"Cell growth gene expression reveals direct fitness costs of grazer induced toxin production in red tide dinoflagellate prey"

The authors present a new way to calculate costs of inducible defense in phytoplankton (toxin production) by using relative gene expression of a mitotic cyclin (cyc) gene. The manuscript is well written and focused. It is definitely of interest for plankton ecologist and is able to motivate more research about new methods determining costs of defenses.

I have a few comments:

-Separating fitness costs of inducible defenses: Such costs are often measured by exposing prey to predators without allowing mortality by grazing. As the defense is inducible, predator related cues may be responsible to induce defenses and no direct contact with predators is necessary. That allows to estimate fitness costs directly by measuring fitness relevant traits such as growth and reproduction without having direct mortality involved. The authors argue that direct predation may result in stronger defense development. However, according to the literature (cited by the authors) supporting this argument a defense in form of toxin production was also measurable by exposing algae just to cues alone. To my opinion a combination of both approaches could have some benefits. In the predator cue treatments one could measure fitness costs of toxin production by directly following fitness relevant parameters (growth) without confounding mortality by grazing. Comparing then toxin production in treatments with only predator cues and treatments with cues and predators would allow to measure differences in toxin production and thereby estimating costs to toxin production also in treatments including mortality by predators.
Thank you for the suggestions. This is somewhat similar to the point brought up by reviewer 1. Point 1 of the addendum explains why we did not try measuring cost in cage experiments as suggested by the reviewer. In the stain used in our study, Both Sent-Batoh (2012) and Park (2018) used cage experiments to show the indirect effects of grazer exposure on toxin production. While the effect is obvious, it is much weaker than direct exposure to grazers. Thus, if a cost were measurable it would not be the real cost of defense in the presence of grazers. Importantly, we think the reviewer is confused in one point of our paper. In the introduction of the manuscript, we had actually said that experiments with cages had trouble detecting a fitness cost of defense. This was reported by references 15,16, and others we now cite in the revised version, including the dissertation of G. Park, the lead author of the manuscript. Nonetheless, we added to the discussion suggesting that further would should look to see if the cost of defense using our approach is also detected in cage experiments (see lines 344-363).

-Estimates from a single gene (cyc) to refer to growth and calculate growth rate reduction and fitness costs. Would this mean that the expression of the gene is mainly linked to somatic growth, but not influenced by any other fitness related parameters?
Our approach uses cell growth as the fitness currency. In phytoplankton individual growth is cell division. There is no somatic growth. We chose to look at the expression of the gene (cyclin B), which is linked to cell division Zhuang et al. (2013).

At the moment, we are cross validating the approach by looking at other possible gene markers related to cell growth; namely, RuBisco (ribulose-1,5-bisphosphate carboxylase/oxygenase); an enzyme that is involved in both carbon fixation and photorespiration at the first step of calvin cycle. *rbcL* has been shown to be a useful target for molecular assays that quantify form- or clade-specific RNA transcript concentrations as a proxy for the carbon fixation activity of marine phytoplankton (John et al. 2007). We

added a brief suggestion in the discussion about cross validating the approach with other markers of phytoplankton functions related to growth (see lines 344-363).

-Toxin production and cyc expression: From figure 2 it looks like that not only the gene expression is different between control and toxin producing phenotypes, but that also the variation around the mean is different (Fig. 2C). Standard deviations are much larger in controls than in treatments. Is this just an effect from less variable growth rates in toxin producing algae? It is difficult to estimate this point from Fig 3 C. Could this otherwise also mean that there is an interaction between toxin production and cyc expression per unit growth? In such a case it would not allow to measure fitness costs by using the expression of cyc, or?

Thank you for the insightful comment. A test did reveal that the standard deviation of *cyc* RGE is larger in the control than in treatment. In retrospect, we realize it was a mistake to put a regression through the control and treatment data in Fig. 3B as this may led readers to think we use that regression to calculate the defense cost. In the revised version of the manuscript we deleted the regression and only report the nonparametric correlation coefficient (Kendall's Tau). It is important, however, to point out that we did not use that earlier regression to calculate the cost of defense. Eq. 7 in the methods section shows that the cost of defense is given by the ratio of RGE *cyc* in the treatment relative to the control. To the extent that RGE of cyc is lower in the treatment than the control, then there is a cost of defense.

-To my opinion one way to control for such an interaction would be to measure cyc gene expression at different growth rates (f.e. along a resource gradient) with and without toxin production and compare the slopes of the regression as seen in Fig. 3A but for both, control and toxin producing phenotypes. Using only the slope of Fig 3A of control animals for cost calculations would mean that the relationship between cyc overexpression based on the reference gene and growth has to be identical between control and toxin producing phenotypes. Is there enough evidence to be sure about that?

Again, see response to the previous comment on how we calculate cost of defense. We did not use the slope of Fig 3A to calculate the cost of defense. Rather, we used Eq. 7, which depends on the RGE of *cyc* in the treatment relative to the control. Details of the calculations were given in Appendix S4.

The reviewer's suggestion is another excellent way to cross validate the proposed approach, and we have added it to the discussion section along with the other suggestions from reviewers 1 and 2 (lines 344-363). These are all future research avenues.

**Addendum for reviewers:**

**1)** Reviewers brought up whether we had done measurements of costs without the direct presence of grazers. We did not do this. Here we present additional information that provides the rationale for the design of our assays.
Previous work from our lab compared induced toxin production and cell growth rate (Fig. 1, from Park 2018) of *Alexandrium catenella* cells in different stages of growth in response to *Acartia hudsonica* copepods (same grazer-to-ratio as in our manuscript). Cells were unexposed (control), in the presence of grazers (direct grazing) and separated from grazers by a mesh (indirect grazing).

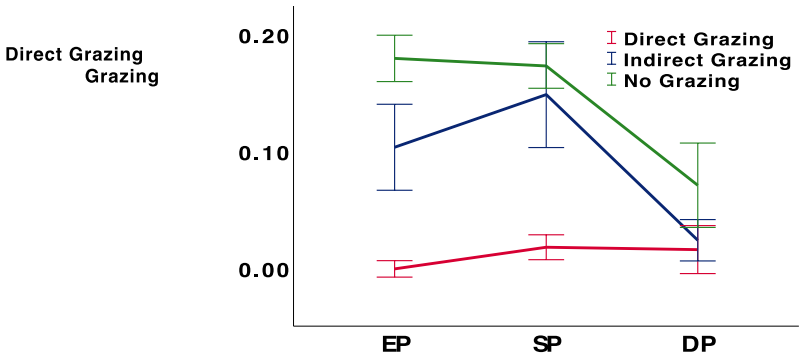


*Figure 1. Cell toxin content (A) and growth rate (B) versus cell growth phase (exponential, stationary, and declining phases) during the grazer-induced toxin assay. Lines represent the treatments (direct and indirect exposure to grazers) and control (no exposure to grazers). Asterisks indicate significant differences among treatments (Post-hoc SNK ANOVA). * = $p < 0.05$; **= $p < 0.01$. Error bars represent ± 1 standard deviation of the mean (N = 3).*

Regardless of growth phase, toxin production significantly increased relative to the control in the direct and indirect exposure to grazer treatments relative to the control. Meanwhile, growth rates significantly decreased relative to the control only in the direct exposure treatment. Growth rates in the indirect treatment were not significantly different from the control in any of the growth phases. This suggests that the cost of toxin production (as evidenced by the indirect treatment) is not apparent (at least during the three-day incubation period of our experiment). This is consistent with that others have found for *Alexandrium* (references cited in the manuscript). The much lower degree of induced defense, and our inability to document a cost of the defense in the indirect treatment were the reason we decided to forego the indirect treatment in the design of experiments for the manuscript under review.

A different kind of experiment, however, did reveal a cost of defense (Fig. 2, from Senft-Batoh 2012). Here, *Alexandrium* cells that previously had been *indirectly exposed* to *Acartia hudsonica* grazers (similar grazer-to-cell ratios and same incubation period as in experiments in Fig. 1) were then grown for **11 days** under either nutrient replete or limited conditions and their growth rates compared to control cells (no exposure to grazers) under the same conditions. The cost of defense is apparent in both the nutrient-replete and nutrient-limited treatments, but less in the latter.

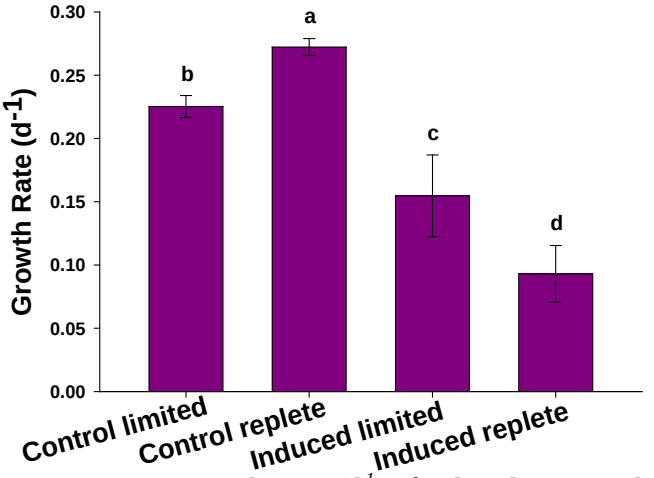


*Figure 2. Mean growth rates ($d^{-1}$) of induced or control Alexandrium catenella in nutrient-replete (F/2) or nutrient-limited (filtered seawater) media, measured over 11 days. The filtered seawater nutrient concentrations were ($[NO_3^-]$= 3 µM; $[PO_4^{3-}]$= 1 µm. A multiple means comparison procedure (post-hoc Student-Newman-Keuls test) indicates that all growth rates are significantly different ($p<0.05$) with a, b, c, d representing the growth rates from highest to lowest, respectively. Bars represent ±1 standard error of the mean (n=10).*

**Conclusion:** A cost of defense is evident for cells exposed to grazer cues but only when growth is measured over longer periods, and that cost is less than when cells experience grazing. Hence, we decided to test for the cost of defense under the presence of grazers. This made the experiments more feasible and also more ecologically relevant.

**2)** Reviewers were concerned about whether RGE *cyc* expression may be also be modified by defense other than toxin production. Figure 3 shows results of an experiment comparing RGE of an STX gene and *cyc* for three *Alexandrium catenella* strains that differed in their toxin content (Park 2018**). The low and moderate strains toxin content is not inducible by the presence of grazers (data not shown). The high toxin content strain, the same used in our manuscript under review, is highly inducible (Park 2018; Griffin et al. 2019; Senft-Batoh et al. 2015a, b).** Constitutive toxin contents (fmol $cell^{-}1$) of the strains are: <5 (low), ~20 (moderate) and > 31 (high). RGE of *sxtA* and cyc were independent of the grazer concentration for the two uninducible strains (low and moderately toxigenic). By contrast, the highly toxigenic stain shows upregulation of *sxtA* with increasing grazing pressure, but downregulation of *cyc*. In our view, this is consistent with the trade-off hypothesis for grazing induced toxigenic defense. At the same time, the data suggests that if defense is other than toxin production, RGE of *cyc* is not affected.

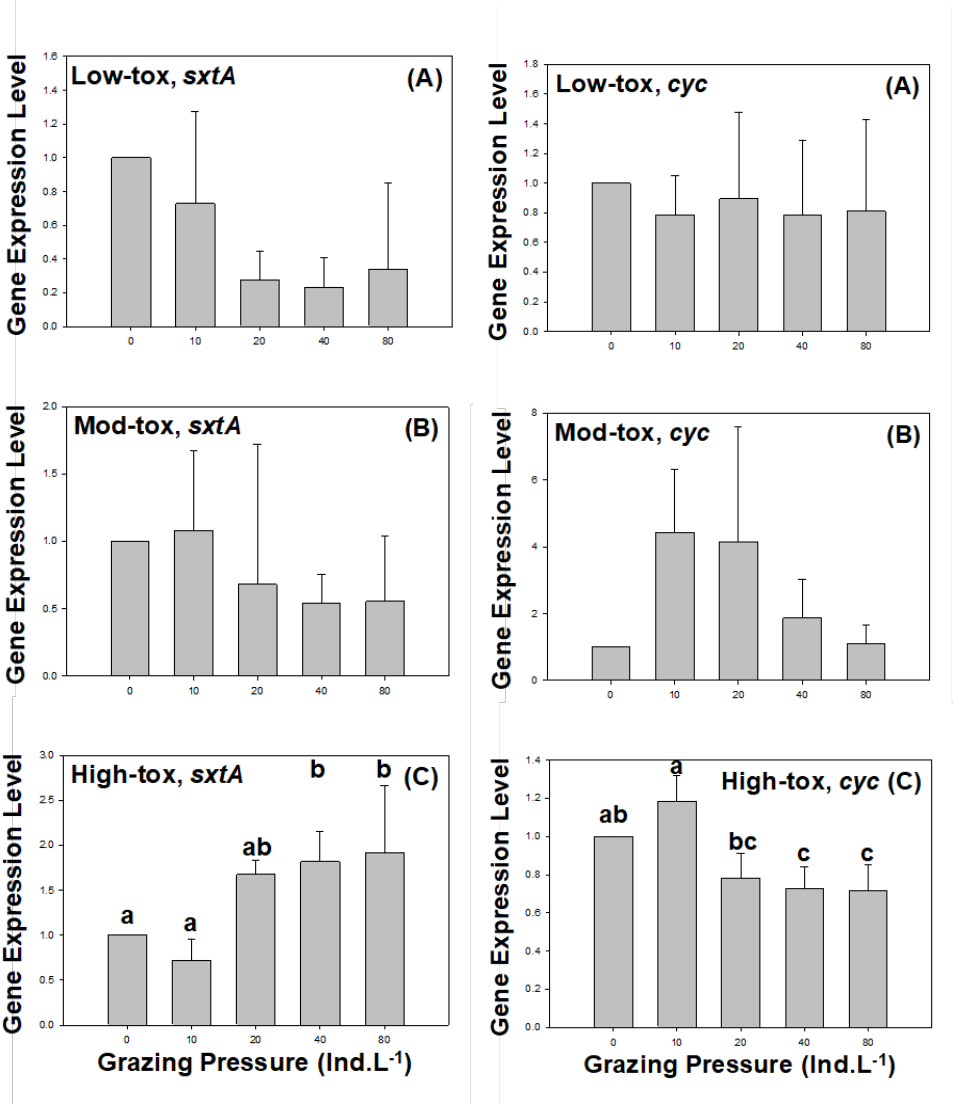


*Figure 3. Relative gene expression of STX gene, sxtA (left panels) and* cell growth-related gene, *cyc*, (right panels) *versus grazing pressure for low (A), moderate (B) and high (C) toxigenic strains of Alexandrium catenella. Letters above bars represent significant differences between mean values of groups compared to the control (no grazing) and also among treatments (p<0.05; ANOVA, post-hoc Tukey HSD). Error bars represent ± 1 standard deviation of the mean (n=3).*

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