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The genetic basis for the evolution of soma: mechanistic evidence for the co-option of a stress-induced gene into a developmental master regulator

Stephan G. König and Aurora M. Nedelcu

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Original submission:	16 June 2020
Revised submission:	1 September 2020
Final acceptance:	9 November 2020

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

Review History

RSPB-2020-1414.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Major revision is needed (please make suggestions 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?
Acceptable

Is the length of the paper justified?
Yes

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

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No

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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
During the evolutionary transition to complex multicellularity, cells differentiated into germ and somatic cells. While the origin of soma is not well understood – theory and recent evidence from several incidences of complex multicellular evolution suggest that the co-option of genes from unicellular organisms is involved. What is nevertheless lacking is the information on pathways and mechanisms underlying a potential gene co-option.
The manuscript investigates the mechanistic underpinning of the evolution of soma in the Volvocine green algae, using the multicellular Volvox cateri as a model. In this species, the differentiated expression of the master regulatory gene regA during development is central for the specialisation into somatic and germ cells. A closely related gene to regA is present in the unicellular relative of Volvox cateri, Chlamydomonas reinhardtii, and is expressed during stress response. With this study the authors aim to differentiate between three possible scenarios for the co-option of the ancestral stress-induced gene into the developmental master regulator regA.

I like the study as it addresses a very timely and relevant topic within the field of multicellularity research, which is also relevant for other evolutionary transitions and complexity evolution. Nevertheless, aspects of the manuscript would benefit from more clarity:

- The non-specialist reader would benefit from an overview on the occurrence of regA, RLS1, rlsD… within the volvocine green algae, for example in form of a table. The current parts in the introduction are full of information that easily gets confusing (line 87 following).

- I might have missed it – but as I see it, the study cannot differentiate between the three postulated scenarios for gene co-option but can only distinguish between two scenarios: A or B+C. This needs to be addressed/ corrected within the abstract, text and as part of Figure 2 – or if I am mistaken, the authors need to spell out how they are going to differentiate between the three scenarios through their experiments – what are the respective expectations.

- To me, it is not clear why the study was designed the way it is. It needs to be clear why that mutant has been used and what the benefit it of not using the wild type as the focal strain. Again, the reader would benefit from clearly linking hypothesis with the experiments and expectations so that the reader is primed.

- How can regA be expressed in the regA- mutant? Please explain and also include in the manuscript.

- DNA laddering is mentioned as the hallmark of PCD. Please briefly explain or at least include a reference to a study that uses this method.

- The authors claim that they provide 'direct evidence' for gene co-option (line 35 and also in the discussion). – I would like to urge them to consider their wording and be more careful with their claim – after all they have been using extant organisms, where this hypothesis cannot directly be tested.

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?
Acceptable

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.
Yes

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> **Is it accessible?**
> N/A
>
> **Is it clear?**
> N/A
>
> **Is it adequate?**
> N/A

Do you have any ethical concerns with this paper?
No

Comments to the Author
See attached. (See Appendix A)

Review form: Reviewer 3

Recommendation
Major revision is needed (please make suggestions 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?
Excellent

Quality of the paper: Is the overall quality of the paper suitable?
Acceptable

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 main aim of König and Nedelcu's manuscript is to gain insight into the molecular / developmental mechanisms of the co-option of RLS1 gene in closely-related unicellular relatives to give rise to regA master developmental regulator gene, key to the evolution of multicellularity –germline and soma– in specific Volvacine lineages, including Volvox carteri. To do this, the Authors used a mutant of V. carteri called dmAMN mutant that is gonadialess, meaning that this mutant suppresses cell type differentiation in germline and soma. The Authors investigate the environmental sensitivity of regA in this mutant in the hope that it will reveal details of the mechanism of it's molecular and regulatory origin in V. carteri. The Authors provide evidence that regA is environmentally inducible in extended dark conditions in the dmAMN mutant and argue that this is an ancestral feature of regA that has been maintained from its ancestor to deal with stressful conditions. This proposes some mechanistic scenarios for the molecular and developmental basis for the origins of multicelullarity in the volvacine lineages leading to V.

carteri.

This is a fascinating system and an important example of multicellularity. I like the approaches and questions asked. I generally found the data solid, but if I have understood this story correctly, then the interpretations of the data weak and potentially need revision to account for the following:

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(2) Furthermore, the Authors found no mutations in the regulatory or intronic regions of the gene, yet the 3 scenarios they are trying to distinguish between in Figure 2 are based on evolved environmental or developmental signals in the regulatory region of regA. To this reviewer, a more parsimonious explanation for the gain of environmental sensitivity of regA is the mutation itself in the regA protein. There are now two clear examples – Eud-1 gene in Pristionchus pacificus worms from Ralf Sommer’s work (Ragsdale et al. 2013) and KCl-1 in C. elegans form the Christian Braendle’s lab (preprint on BioRXiv) – showing that mutations in protein coding regions of key genes influence the environmental sensitivity of regulatory genes and development.

(3) If it is the protein-coding mutation itself that increases environmental sensitivity, then the environmental sensitivity could have evolved from the ancestor and lies latent or dormant in regA and the protein-coding mutation released this ancestral genetic potential. Please see Mary-Jane West Eberhard 2003 Book, Suzuki and Nijhout 2006 Science, Rajakumar et al. 2012 Science, for discussion and examples of ancestral developmental potentials and enabling mutations and environmental stress.

(4) Finally, the Authors must take into account these 3 above points in building and testing their possible models and scenarios in Figure 2, and revise accordingly, to change or broaden their interpretations of their data, which at present I find speculative in light of the evidence I present above.

Decision letter (RSPB-2020-1414.R0)

12-Aug-2020

Dear Dr Nedelcu:

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 Editors have raised some concerns 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.

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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. Please see our Data Sharing Policies (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.

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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].

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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,
Dr Maurine Neiman
mailto: proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
The reviewers are all of the view that this is a strong MS, and also that it needs strengthening in key areas of explaining the logical connections between hypotheses to be tested and experiments performed, and in the interpretation of these data on an extant organism as suggesting specific scenarios for previous evolutionary events. I concur with these major areas of revision.

Reviewer(s)' Comments to Author:
Referee: 1

Comments to the Author(s)
During the evolutionary transition to complex multicellularity, cells differentiated into germ and somatic cells. While the origin of soma is not well understood – theory and recent evidence from several incidences of complex multicellular evolution suggest that the co-option of genes from unicellular organisms is involved. What is nevertheless lacking is the information on pathways and mechanisms underlying a potential gene co-option.
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Referee: 2
Comments to the Author(s)
See attached

Referee: 3
Comments to the Author(s)
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Author's Response to Decision Letter for (RSPB-2020-1414.R0)

See Appendix B.

RSPB-2020-1414.R1 (Revision)

Review form: Reviewer 1

Recommendation
Accept as is

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

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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
I am happy with the revision of the ms. The authors addressed all comments to my satisfaction.

Decision letter (RSPB-2020-1414.R1)

09-Nov-2020

Dear Dr Nedelcu

I am pleased to inform you that your manuscript entitled "The genetic basis for the evolution of soma: Mechanistic evidence for the co-option of a stress-induced gene into a developmental master regulator" 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.

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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,

Dr Maurine Neiman
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Appendix A

The evolution of germ-soma cellular differentiation is a critical step in the evolution of multicellular complexity and individuality. The volvocine green algae are a well-known model system for studying the development and evolution of cellular differentiation and are the model system used in Manuscript RSPB-2020-1414. Specifically, the authors describe the gene expression patterns of *regA*, a key somatic cell differentiation regulatory gene, in both wild-type *V. carteri* and a cellular differentiation lacking mutant under a variety of conditions. The objective of this study is to test a popular hypothesis that the genetic basis for cellular differentiation was co-opted from stress response genes present in the unicellular ancestor of multicellular species. This hypothesis proposes that the regulation of *regA* underwent a shift from environmental control to developmental control, but the authors seek to test a version of this transition where *regA* maintains some environmental regulation. Overall, the authors present a clear study that supports the hypothesis that *regA* retains some ancestral environmental regulation and may play a role in stress tolerance. These results represent an important step in better understanding how the co-option from environmental to developmental regulation of a key cellular differentiation regulatory gene occurred in the volvocine green algae. Likewise, the general approach of the paper can serve as a template for further testing of the hypothesis using different forms of stress. However, the authors need to more clearly discuss why developmental regulation alone is not sufficient to explain their results.

Major comments

The use of light and dark cycles to test environmental regulation of *regA* presents a challenge to the interpretation the authors results: the development of *Volvox* is directly effected by light/dark cycles. The authors sought to determine if *regA* exhibits environmental regulation in addition to its already well characterized developmental regulation. Supporting this claim also means demonstrating that their results cannot be explained by developmental regulation alone. But the choice to manipulate an environmental variable that also directly affects developmental timing and progression makes accomplishing this task challenging. This problem is less extreme in *V. carteri* than other *Volvox* species that rely on light cues to time cell divisions. Nevertheless, the authors should explicitly discuss that developmental regulation alone cannot explain their results.

Indeed, on my first read through of the manuscript I thought their expression results could be explained by the dmAMN mutants attempting to restore normal *development* after experiencing a *developmental* perturbation caused by a prolonged dark period rather than as a direct response to environmental stress. Under this developmental disruption hypothesis the mutant's increase in expression of *regA* following a prolonged dark period and reintroduction to light could be explained as an attempt by the organism to reset its developmental clock following perturbation. And the relative lack of increased *regA* expression in the WT could be explained by the fact *regA* is functional in the WT but not the mutant. On my second read through I realized that the experiment reported in Figure S7 refutes this idea because it shows upregulation of *regA* in response to perturbation at different developmental time points. But it shouldn't take two careful read throughs and a look at the supplemental materials for a reader to be convinced that the clear alternative hypothesis (i.e. developmental regulation only) to the author's primary hypothesis (i.e. developmental + environmental regulation) is not supported. The fix is fairly simple, a few more sentences in the discussion (i.e. section 4a) and perhaps moving Figure S7 into the main text.

Minor comments

1. In the introduction, the authors cite Matt and Umen 2018, a transcriptome analysis of germ cells and somatic cells in Volvox carteri. But they never discuss that a key finding of this paper is that expression of orthologs of genes upregulated in the dark in *Chlamydomonas* are enriched in *V. carteri* somatic cells. While previous work by Nedelcu on *RLS1* was the primary inspiration for the present study, the relationship between temporal and spatial regulation shown by Matt and Umen further places the results of the current manuscript in a genome-wide context.
2. The authors describe the dmAMN mutant used in this paper as a *glsA⁻/regA⁻* double mutant. They describe the *regA* mutation in this mutant but never the *glsA* mutation. Ideally, the authors should describe both gene mutations. But the phenotype of dmAMN is consistent with known *glsA⁻/regA⁻* mutants so this issue is relatively minor. Still, if the authors choose not to or are unable to describe the *glsA* mutation, then they should explicitly acknowledge that description of the *glsA⁻* mutation is based on phenotype alone.
3. In section 2c, the PCR machine used should be listed in addition to the software used.
4. In the paragraph in lines 166 – 174, the authors begin describing the *regA* mutation in dmAMN then suddenly switch to a different strain (HB11A) then back to dmAMN. This is jarring and hard to follow. To simplify I suggest describing dmAMN completely first and then compare to HB11A.
5. In section 3b, the authors propose two hypotheses for why the expression of *C. reinhardtii's RLS1* gene is different than *V. carteri's regA* gene following a prolonged dark period. But there is a third possibility as well, cross-talk between the developmental and environmental regulatory pathways of *regA*. In other words, the developmental regulation of *regA* may modify how environmental stimuli affect *regA* expression compared to *RLS1*.
6. Figure 4b, what the black and white lines represent on the graph should be explicitly stated in the figure legend.
7. Many figures throughout the manuscript describe relative differences. But it is not always clear what the conditions shown are relative to. Making sure this is clear in every figure legend, and if applicable adding an inset to the graphs stating what the reference condition is would make the many figures much clearer.
8. Figure 5d reports the results of a Tukey's HSD test, which is a multiple comparisons test where all conditions (in this case dark, 20, 70, 260) are compared to each other. The authors report statistically significant differences, but it is not clear which categories are different from each other and if any categories are not significantly different from each other.
9. The supplemental methods seem to have an issue with some citations. In several places a reference is listed as "Error! Reference source not found." This is likely due to an issue with whatever citation management software the authors are using. Regardless, this and typos throughout the whole manuscript, such as use of "ii" when meaning "iii" in the abstract, among others, should be fixed.
10. In supplemental methods, section 1b that authors describe using a pestle to separate *Volvox* colonies, but not the apparatus the pestle was used in. I assume it was a dounce homongenizer but they should say so.

Appendix B

Supplementary Material

1. Material and Methods

(a) Strains and culturing conditions

The two *V. carteri* strains used in this study were: a female wild type strain (known as EVE; [1] and a male gonidialess strain (dmAMN). The latter is a spontaneous gonidialess mutant isolated in our lab from the *regA*⁻ strain UTEX1877. Under standard culturing conditions, synchronous cultures of both strains were grown in aerated standard *Volvox* medium (SVM; [2]) at 30°C (EVE) or 32°C (dmAMN) and a photoperiod of 16L:8D. Light was provided by cool white fluorescent tubes at 260 μmol photons m^{-2} s^{-1} photosynthetically active radiation (Figure S1)—as measured with a Laboratory Quantum Scalar Irradiance Meter QSL-100 (Biospherical Instruments Inc.). Under these conditions, the generation time was 48 h for EVE and 72 h for dmAMN. Stock cultures were transferred every generation at the start of embryogenesis during the light period (the 0 h time point, Figure 1), and grown in 300 mL SVM with starting densities at 0.625 spheroids/mL for EVE or 0.67 spheroids/mL for dmAMN.

(b) Isolation of early EVE embryos

EVE spheroids containing 2-celled embryos (defined as 0 h time point, Figure 1*a*) were collected on a 100 μm Nitex filter and resuspended in 40 mL fresh SVM in a 40 mL dounce homogenizer (Kontes) at ≤ 300 spheroids/mL density. Spheroids were broken with one down-and-up stroke of the loose-fitting pestle A of the dounce homogenizer, and the cell suspension was incubated for 10 min at room temperature so embryos could fully dissociate from the somatic cell sheets. The suspension was poured on a 100 μm Nitex filter; the embryos passed through the filter in the flow-through, were collected on a 30 μm Nitex filter, washed with 100 mL SVM to remove any loose somatic cells, and used to start the experimental cultures.

(c) Experimental cultures under different light regimes

Experimental cultures were started at the 1 h time point (after the onset of embryogenesis) and grown in 150 mL SVM at densities of 25–60 embryos/mL for EVE and 5–15 spheroids/mL for dmAMN. At the end of the first light period (time point 2 h) one set of cultures were switched to dark for various lengths and then exposed to light for different periods of time; the other set remained in the dark for the total amount of time. For mature dmAMN with large cells, cultures were first grown under the standard light conditions of 16L:8D for an additional 24 h before being switched to dark. Light intensity was manipulated by wrapping culture flasks in black fiberglass screen and by placing them at various distances from lamps. To adjust light quality, culture flasks were wrapped in red, green or blue filters (#027, 735, and 071 respectively, LEE Filters; Figure S1); the transmitted photosynthetically active radiation was 5 μmol photons m^{-2} s^{-1}.

(d) RNA extraction and quantification

dmAMN or EVE cultures were collected on a 30 μm Nitex filter and resuspended in 1 mL fresh SVM in a microcentrifuge tube; the sample was centrifuged for 1 min at

3000 g at room temperature, the supernatant discarded, and the pellet flash-frozen in liquid nitrogen. RNA was extracted from up to 200 mg of material with the RNeasy Plant Mini Kit (Qiagen) using Buffer RLC, and eluted in 50 µL ddH_2O. RNA concentration was determined either with a NanoVue (GE) or a SpectroMax M5 spectrophotometer (Molecular Devices). RNA integrity was tested on RNA 1X SB gels (5 mM disodium borate decahydrate, pH adjusted to 8.5 with solid boric acid) containing 1.2% agarose, 20 mM guanidine thiocyanate and 0.5X SYBR Safe (Invitrogen; [3,4]). To remove any RNA secondary structures prior to electrophoresis, the RNA samples were added to 1X Blue Gel Loading Dye (NEB) and 20 mM guanidine thiocyanate in a total volume of 15 µL, incubated for 10 min at 70°C, and then cooled on ice for 2 min. Equal RNA amounts were used for all samples within one experiment. To remove genomic DNA contamination, up to RNA samples were treated with RQ1 DNase (Promega). Reverse transcription reactions were performed with SuperScript III Reverse Transcriptase (Invitrogen) and either 2.5 µM oligo$(dT)_{20}$ for reference gene *rps18* or 0.1 µM gene-specific primer for gene of interest *regA* (Table S1) in 10 µL total volume.

(e) Quantitative real-time PCR

Each reaction contained 2 µL template in 1X KAPA SYBR FAST Master Mix Universal (Kapa Biosystems), and 0.2 µM of each primer (Table S1). The PCR run was performed in a Rotor-Gene 6000 (Corbett Research) with an initial denaturation at 95°C for 3 min followed by either 40 (*rps18*) or 45 (*regA*) PCR cycles (95°C/3 sec; 55°C/30 sec; 72°C/3 sec), and concluded by a melt curve analysis from 72 to 95°C. Two +RT replicates and one −RT for each biological replicate and a no template control (NTC) were included in each run. Specific amplification of the *regA* locus was confirmed by sequencing of the PCR product (Figure S2). Data was collected with the Rotor-Gene 6000 software (Version 1.7.87, Corbett Research) and exported to LinRegPCR (Version 12.17; [5][6]) to calculate average PCR efficiency and threshold cycle (C_T); C_T for technical replicates was averaged. The relative expression of the gene of interest between samples was determined with REST2009 software (Version 2.0.13, Qiagen); the software also determined if differences between the control and treatment group were significant using a randomization test.

(f) Cell viability

Cultures were collected on a 30 µm Nitex mesh and resuspended in 1 mL fresh SVM in a microcentrifuge tube. SYTOX Green (Invitrogen) was added at a final concentration of 1 µM and samples were incubated for at least 5 min in the dark [7]. Spheroids were observed on a glass slide with a Leica DM R upright microscope and Leica DC 500 digital camera using the Leica fluorescence FITC filter. Live and dead cells on one hemisphere of at least 20 spheroids per technical replicate were counted using the Fiji image processing package and the cell counter plug-in. Statistical analysis was performed with JMP (Version 10, SAS).

(g) Genomic DNA extraction

To test for DNA laddering (a general feature of programmed cell death that was previously observed in *V. carteri* [8], genomic DNA was phenol extracted from about $1.2–2 \times 10^7$ cells per sample and run on a 1X SB gel (5 mM disodium borate

decahydrate, pH adjusted to 8.5 with solid boric acid) containing 2% agarose [4] and 0.5X SYBR Safe (Invitrogen).

(h) Sequencing of dmAMN *regA* locus

Sequences of the dmAMN *regA* locus were amplified (Table S2) and PCR products were sequenced at the McGill University and Génome Québec Innovation Centre using a 3730x DNA Analyzer (Applied Biosystems).

(i) Determination of a suitable reference gene

To select the most stable reference gene for *regA* expression studies the following candidate genes were investigated: ribosomal protein 18 (*rps18*, GenBank# XM_002946177), TATA-box binding protein (*tbpA*, GenBank# AY787798), and actin (*actA*, GenBank# M33963). Synchronous cultures of EVE were grown in SVM at 30°C in a photoperiod of 16L:8D. At −6 h (6 hours before the onset of embryogenesis—used as time 0), cultures were diluted to 40 spheroids/mL in a final volume of 40 mL and either maintained in 16L:8D or switched to dark at the end of the current light period. Samples were collected at time points 9 h and 15 h, and RNA was extracted as described above. To remove any gDNA contamination, 670 ng RNA were treated with 2 U DNase I, Amp Grade in 1X DNase I Reaction Buffer (Invitrogen) in a total volume of 20 µL for 15 min at room temperature; the reaction was stopped by adding 2 µL of 25 mM EDTA and a 10 min incubation at 65°C, and then kept on ice. cDNA was synthesized from 4 µL DNase-treated RNA using 15 U ThermoScript RT and 40 U RNase Out in 1X cDNA Synthesis Buffer (all Invitrogen), 1 mM each dNTPs, 5 mM DTT, and 2.5 µM oligo(dT)$_{20}$ in 20 µL total volume; the reaction was incubated for 1 h at 60°C, and then terminated for 5 min at 85°C. The newly synthesized cDNA was further treated with 4 U RNase H (Invitrogen) for 20 min at 37°C. qPCR (using additional primers listed in Table S3) and data analysis to calculate C_T was performed as described above, then gene stability was determined with BestKeeper and geNorm [9,10]. The *rps18* was chosen as reference gene for both its stability (Table S4) and its prior use as reference gene for *V. carteri*, in particular for the initial *regA* expression studies [11–15].

(j) Sequencing of qPCR products

regA qPCR products were first purified by gel-electrophoresis and gel-extraction, and then subcloned with the CloneJET PCR Cloning Kit (Thermo Scientific); *regA* clones and *rps18* qPCR products were then sequenced (for *regA* see Figure S2, for *rps18* data not shown) as described above.

(k) Cell viability

Effect Tests (JMP 10 Software) were performed to determine which independent variable most likely caused the difference in dmAMN cell viability data; the tests indicated that the differences in cell death levels observed among treatments are most likely explained by absence/presence of light (Table S5A) and cell size (Table S5B). Light did not cause a significant increase in somatic cell death in early EVE juveniles (Table S6A). The presence of light and the phenotype (dmAMN vs. EVE) alone most likely explains the response in the level of cell death (Table S6B).

Supplementary Tables

Table S1. Gene-specific primer (GSP) for reverse transcription and qPCR primers (F, forward; R, reverse)

Region*	Primer sequences (5' to 3')
rps18† (+225 to +362 bp)	F TCGCGCTTACAAGATTCCG R TGGTTGCGGATCTTCTTCAG
regA‡ (+1503 to +1603 bp)	GSP CGACGCTCCTGTCGAGGC F CAATGGCAGCAAATGGATGTC R GTTCCAAATCAGGCAACACG

all oligonucleotides were provided by Sigma Genosys
*relative to cDNA position
†primers designed with IDT SciTools PrimerQuest program, IDT, Coralville, USA. Retrieved 12 December, 2012. http://www.idtdna.com/Scitools.
‡GSP from [16]; F & R from [17].

Table S2. PCR primers and conditions for amplification of *regA* sequences.

All PCRs were run in a RoboCycler Gradient 96 (Stratagene); additional primers used for sequencing of PCR products are also listed; F, forward primer; R, reverse primer; SP, sequencing primer.

regA region*	Primer sequences (5' to 3', artificial sequences are in red†) and conditions
Promoter–Exon 5 (−125 to +2498 bp)	F CCTGTCCGTATCTGGCATTGG R CTCCTGTCCCAATCACGCAGG 50 µL PCR mix: 0.2 µM each Primer, Platinum PCR SuperMix High Fidelity PCR program: 94°C/2 min—[94°C/0.5 min; 55°C/0.5 min; 68°C/2 min]×45—68°C/10 min SP GGATTGAGAATCGCCATTTCG attB1 SP GGGGACAAGTTTGTACAAAAAAGCAGGCTGTAAGCCTGTTCCGCTTCC attB2 SP GGGGACCACTTTGTACAAGAAAGCTGGGTCTGCAGACAATGCAAAAAGC
Part of Intron 5 (+2885 to +3830 bp)	attB1 F GGGGACAAGTTTGTACAAAAAAGCAGGCTCATTGATATCAGATGTAAC attB2 R GGGGACCACTTTGTACAAGAAAGCTGGGTGGATATCCGATTGAGGCAGG 50 µL PCR mix: 0.2 µM each Primer, 1 U *Taq*, 1X HB, 2 mM $MgSO_4$, 0.2 mM each dNTPs PCR program: 94°C/2 min—[94°C/0.5 min; 50°C/1 min; 68°C/2 min]×45—68°C/10 min
Part of Exon 6‡ (+4860 to +5609 bp)	F TTGGGAGCCGACCTGCCAT R AGCGTGACCTCGCATGTATC 50 µL PCR mix: 0.2 µM each Primer, 1 U *Taq*, 1X AB, 1X ES, 1.5 mM $MgSO_4$, 0.2 mM each dNTPs PCR Program: 94°C/2 min—[94°C/0.5 min; 55°C/1 min; 68°C/2 min]×45—68°C/10 min
Intron 7 (+7280 to + 8539 bp)	attB1 F GGGGACAAGTTTGTACAAAAAAGCAGGCTGGCGGCTCTTCCGGCAGTG attB2 R GGGGACCACTTTGTACAAGAAAGCTGGGTTGCGGAACCTGCGACGGTG 50 µL PCR mix: 0.2 µM each Primer, 2.5 U *Taq*, 1X AB, 1X ES, 1.5 mM $MgSO_4$, 0.2 mM each dNTPs PCR program: 94°C/2 min—[94°C/0.5 min; 55°C/1 min; 68°C/2 min]×45—68°C/10 min SP CTCCTGCAACAGCACCAACG SP CCTGAAGGTCAAGGTGAAGC

Taq, Platinum *Taq* DNA Polymerase High Fidelity; HB, High Fidelity PCR Buffer; AB, PCRx Amplification Buffer; ES, PCRx Enhancer Solution (all reagents Invitrogen); all oligonucleotides were provided by Sigma Genosys
*relative to gDNA
†Artificial sequences in primers were not relevant in this study
‡Primers from [16]

Table S3. Additional qPCR primers (F, forward; R, reverse) for the amplification of *tbpA* and *actA*.

Region*		Primer sequences (5' to 3')
tbpA† (+531 to +636 bp)	F	TGCTCATCTTTGTGTCGGG
	R	TGCGTCTCCTTTCTTGTACTG
actA‡ (+560 to +867 bp)	F	TGACGGACTACCTGATGAAG
	R	GACATCGCACTTCATGATGC

all oligonucleotides were provided by Sigma Genosys
*relative to cDNA position
†primers designed with IDT SciTools PrimerQuest program, IDT, Coralville, USA. Retrieved 12 December, 2012. http://www.idtdna.com/Scitools.
‡from [18]

Table S4. Gene stability of *rps18*, *tbpA* and *actA* for selected time points.

	rps18	*tbpA*	*actA*
C_T of samples @			
9 h	16.158	26.206	18.834
15 h (16L:8D)	16.331	26.724	17.814
15 h (cont. dark)	16.357	27.547	20.399
Gene stability (the lower the more stable)*			
BestKeeper†	0.067	0.467	0.933
geNorm‡	0.557	0.557	0.925

*using RefFinder (http://www.leonxie.com/referencegene.php)
†[10]
‡

Table S5. Light influences the viability of dmAMN cells.

Source	DF	F Ratio	Prob > F
(**A**) Effect test of light intensity on cell death of small cells (Figure 5b)			
individual [technical replicate, biological replicate]	202	0.8521	0.9109
technical replicate [biological replicate]	6	0.53	0.7856
biological replicate	2	0.1527	0.8584
light intensity	3	64.1825	< 0.0001
(**B**) Effect test of cell size and light on cell death (Figure 5*b* and S8B)			
individual [technical replicate, biological replicate]	242	0.9768	0.5801
technical replicate [biological replicate]	6	0.6134	0.7197
biological replicate	2	1.1391	0.3208
light	1	236.5805	< 0.0001
cell size	1	6.5005	0.011
light*cell size	1	31.7315	< 0.0001

Table S6. Phenotype and light affect viability of *V. carteri* cells.

Source	DF	F Ratio	Prob > F
(**A**) Effect of light on cell death of EVE cells (effect test for data in Figure 7*b*)			
individual [technical replicate, biological replicate]	173	1.0685	0.3301
technical replicate [biological replicate]	6	0.0328	0.9998
biological replicate	2	0.0033	0.9967
light	1	3.7757	0.0536
(**B**) Effect of light and phenotype on cell death (effect test for data of small dmAMN cells and EVE in Figure 5*b* and 7*b*)			
individual [technical replicate, biological replicate]	194	1.0833	0.2419
technical replicate [biological replicate]	6	0.4671	0.8328
biological replicate	2	0.3563	0.7005
light	1	129.926	< 0.0001
phenotype	1	26.4331	< 0.0001
light*cell phenotype	1	120.9234	< 0.0001

Supplementary Figures

Figure S1

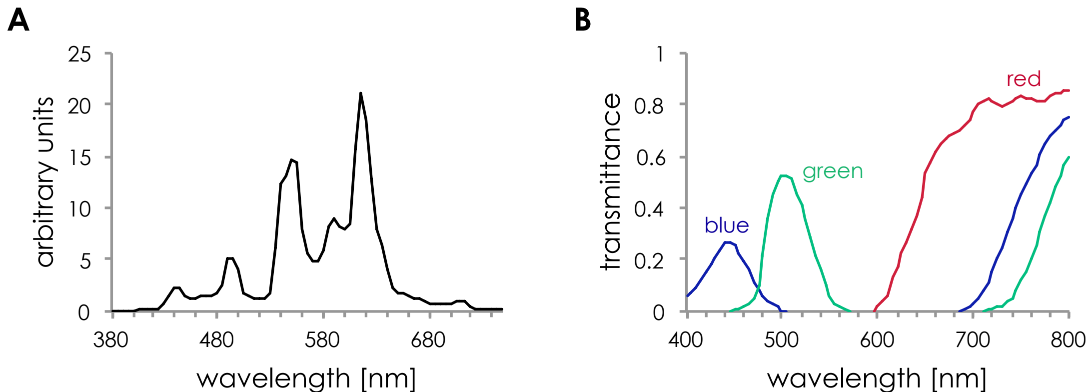

Figure S1 (A) Cool white light spectrum and (B) transmittance of red, green or blue filter (#027, 735, and 071 respectively, LEE Filters).

Figure S2

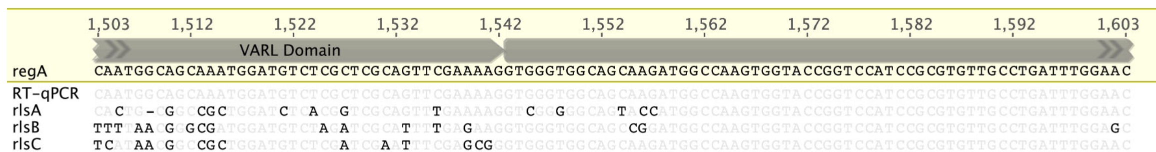

Figure S2. *regA* RT-qPCR specifically amplifies the *regA* locus and not other homologs in the *VARL* gene family.

The cDNA sequences encoding the VARL domain (a putative DNA binding domain) of *regA* and its three closest homologs, *rlsA*, *rlsB*, and *rlsC* were aligned using ClustalW [19] as part of Geneious 6.0 (Biomatters); base numbers are given relative to the *regA* cDNA, the exon border is after 1542 bp. The sequenced *regA* RT-qPCR product is identical to the *regA* sequence only.

Figure S3

Figure S3 The *regA* locus of dmAMN contains an insertion in exon 6. Part of *regA* exon 6 is shown and the EVE sequence is given as wild-type reference. dmAMN is a derivative of UTEX LB 1877 and their *regA* locus contains a 365 bp insertion (green bar) after position +5222 relative to transcription start site; the insertion contains a premature stop codon (indicated in blue) and the VARL domain (red bar) is not translated. For comparison the deletion in the *regA* locus of the *regA* mutant strain HB11A and its derivates is also given (black bars); the deletion causes a frame shift mutation resulting in a premature stop codon and the VARL domain is not translated either [11].

Figure S4

Promoter

```
       -103  -94  -84  -74  -64  -54  -44  -34  -24  -14  -1
                                             Promoter
EVE    ATAAATGCGTACAAGCCAATGCAAGAACGTACATTGTAACTTGTTGTACATTGTCGTTGCGTTGGAGACCCTTCCTAATTGAACTGTTTAAACAATATGGAGC
dmAMN  ATAAATGCGTACAAGCCAATGCAAGAACGTACATTGTAACTTGTTGTACATTGTCGTTGCGTTGGAGACCCTTCCTAATTGAACTGTTTAAACAATATGGAGC
```

Intron 3

```
       1,229  1,238  1,248  1,258  1,268  1,278  1,288  1,298  1,308  1,318  1,328
                                  Intron 3
EVE    GTAAGCCTGTTCCGCTTCCTAACGGACCTCCAGGTACCTGTGTGTGGGGCTCATGACAGCTCTTTTGGATGTTAGAGACTAGCATCTTGCAGCAAGGGCATTC
dmAMN  GTAAGCCTGTTCCGCTTCCTAACGGACCTCCAGGTACCTGTGTGTGGGGCTCATGACAGCTCTTTTGGATGTTAGAGACTAGCATCTTGCAGCAAGGGCATTC
       1,338  1,348  1,358  1,368  1,378  1,388  1,398  1,408  1,418  1,428
                                  Intron 3
EVE    AGTATGAACTAGTAGCATTCTGTAAGAATCTAGGCGGTGCACATGCTGCGCGGACTAACAATCCTTGGGCCGCCTTCCTGGCCGTTTGTTTGGGCTTGTCCTC
dmAMN  AGTATGAACTAGTAGCATTCTGTAAGAATCTAGGCGGTGCACATGCTGCGCGGACTAACAATCCTTGGGCCGCCTTCCTGGCCGTTTGTTTGGGCTTGTCCTC
       1,438  1,448  1,458  1,468  1,478  1,488  1,498  1,508  1,518  1,528
                                  Intron 3
EVE    TGCCAAAGCGCGCACCTTCTCTTGCTTATCGAGGCGGGCTTAAGCTCAAGCTTAGGCTTAGGTCAAAATAACGCAGGCTCAGGGACCTGCAAAAGCATTTGCT
dmAMN  TGCCAAAGCGCGCACCTTCTCTTGCTTATCGAGGCGGGCTTAAGCTCAAGCTTAGGCTTAGGTCAAAATAACGCAGGCTCAGGGACCTGCAAAAGCATTTGCT
       1,538  1,548  1,558  1,568  1,578  1,588  1,598  1,608  1,618  1,628  1,638
                                  Intron 3
EVE    CGAGAGAATCTTGGTGCTATTTTCCGGCGCCACTGAAGGCTTTCTCTCAAAACCGGCGTGCTCATGCCAAGCGCTGTGAAAACATTTTTTCCTGATCTCCCTT
dmAMN  CGAGAGAATCTTGGTGCTATTTTCCGGCGCCACTGAAGGCTTTCTCTCAAAACCGGCGTGCTCATGCCAAGCGCTGTGAAAACATTTTTTCCTGATCTCCCTT
       1,648  1,658  1,668  1,678  1,688  1,698  1,708  1,718  1,728  1,738
                                  Intron 3
EVE    ACAGCTGTACAAATAGCTCCGTCCGCTGCGCTTCTTTTTCAGACGTCGCGCCCTCATATCCTCCAACCTTTTCAGCCGCAAGCTTGATCCCAGACGCACGCGT
dmAMN  ACAGCTGTACAAATAGCTCCGTCCGCTGCGCTTCTTTTTCAGACGTCGCGCCCTCATATCCTCCAACCTTTTCAGCCGCAAGCTTGATCCCAGACGCACGCGT
       1,748  1,758  1,768  1,778  1,788  1,798  1,808  1,818  1,828  1,838
                                  Intron 3
EVE    TCATTCCTTCCTTTCTGGTTGTTGCAAATACTGACGCAGTCACCACAGATGCCTTTAAAACTGCCCAACCCGACGGCACCCTGGCACTCCACCTTCACACCAC
dmAMN  TCATTCCTTCCTTTCTGGTTGTTGCAAATACTGACGCAGTCACCACAGATGCCTTTAAAACTGCCCAACCCGACGGCACCCTGGCACTCCACCTTCACACCAC
       1,848  1,858  1,868  1,878  1,888  1,900
                    Intron 3
EVE    CGCAGCCATTGCCGACTTTGTGTTCCTACCTTGTGCTTTTTGCATTGTCTGCAG
dmAMN  CGCAGCCATTGCCGACTTTGTGTTCCTACCTTGTGCTTTTTGCATTGTCTGCAG
```

Part of Intron 5

```
       2,892  2,901  2,911  2,921  2,931  2,941  2,951  2,961  2,971  2,981  2,991
                       Part of intron 5 (EcoRV to EcoRV)
EVE    ATCAGATGTAACAAATTAATTAAAATGCATTAGTCCGAGCGATTGCCTGTCCGTAACGCAGTCGAATTTCTTAGTGCAAACCGATAGAGATTTAGGATAAAGC
dmAMN  ATCAGATGTAACAAATTAATTAAAATGCATTAGTCCGAGCGATTGCCTGTCCGTAACGCAGTCGAATTTCTTAGTGCAAACCGATAGAGATTTAGGATAAAGC
       3,001  3,011  3,021  3,031  3,041  3,051  3,061  3,071  3,081  3,091
                       Part of intron 5 (EcoRV to EcoRV)
EVE    GCACGTTGAAAGTGCATTTCTTAAATTTGCCATGTTCACTGCATCATATCATCGCTTAGTTTGAATGAGCGGATGCGGGCGAAGGCGACCCATATCGGTGTAT
dmAMN  GCACGTTGAAAGTGCATTTCTTAAATTTGCCATGTTCACTGCATCATATCATCGCTTAGTTTGAATGAGCGGATGCGGGCGAAGGCGACCCATATCGGTGTAT
       3,101  3,111  3,121  3,131  3,141  3,151  3,161  3,171  3,181  3,191
                       Part of intron 5 (EcoRV to EcoRV)
EVE    CGCGGTTGCGCGACGCATATCAAAGCATTATCCATACCTGGTCTCAGGTGATATATTTCGAATGATAGAGATTACATTACATGCCGTTGCGTACACACCATAA
dmAMN  CGCGGTTGCGCGACGCATATCAAAGCATTATCCATACCTGGTCTCAGGTGATATATTTCGAATGATAGAGATTACATTACATGCCGTTGCGTACACACCATAA
       3,201  3,211  3,221  3,231  3,241  3,251  3,261  3,271  3,281  3,291  3,301
                       Part of intron 5 (EcoRV to EcoRV)
EVE    CGATGCTTACCTGTAACATTGGTGCTTACTAACAACGCATTCATCGAACGGCAATTTAGATCGCTTACGGTAAGCACGCTGTGCCAGAAACATCATTCAACGT
dmAMN  CGATGCTTACCTGTAACATTGGTGCTTACTAACAACGCATTCATCGAACGGCAATTTAGATCGCTTACGGTAAGCACGCTGTGCCAGAAACATCATTCAACGT
       3,311  3,321  3,331  3,341  3,351  3,361  3,371  3,381  3,391  3,401
                       Part of intron 5 (EcoRV to EcoRV)
EVE    GAAATCGCTAAACCGCTTGCTTAGGTTTGAAAGTGTCCTCCGAACGGAATTTCCGCTGTTCCGCAGCGACACACGCGAAAGTTTCCCACTTCTCCCCCAACAG
dmAMN  GAAATCGCTAAACCGCTTGCTTAGGTTTGAAAGTGTCCTCCGAACGGAATTTCCGCTGTTCCGCAGCGACACACGCGAAAGTTTCCCACTTCTCCCCCAACAG
       3,411  3,421  3,431  3,441  3,451  3,461  3,471  3,481  3,491  3,501
                       Part of intron 5 (EcoRV to EcoRV)
EVE    TTTTTGTGGTCAACGTCGAAACGCGCAAGTCATGCATTAACAAATAACCTTTGGAAAGAAATAAACCTCAAATGGCCGGGATCCACAGACCGCGCACAGGATC
dmAMN  TTTTTGTGGTCAACGTCGAAACGCGCAAGTCATGCATTAACAAATAACCTTTGGAAAGAAATAAACCTCAAATGGCCGGGATCCACAGACCGCGCACAGGATC
       3,511  3,521  3,531  3,541  3,551  3,561  3,571  3,581  3,591  3,601  3,611
                       Part of intron 5 (EcoRV to EcoRV)
EVE    ATCCGTAAGGCTAGTGCAGATTCGACGGCCGAGGCGGTTCCGAAGGCACAGGAGTTACTTCCGGGTTACCGCGTTCCCTTCCGAGGCACGCAGACTGTAACTG
dmAMN  ATCCGTAAGGCTAGTGCAGATTCGACGGCCGAGGCGGTTCCGAAGGCACAGGAGTTACTTCCGGGTTACCGCGTTCCCTTCCGAGGCACGCAGACTGTAACTG
       3,621  3,631  3,641  3,651  3,661  3,671  3,681  3,691  3,701  3,711
                       Part of intron 5 (EcoRV to EcoRV)
EVE    CAACAGGGAAAAGCAAATCAAAAGTGCAGGAGCACGGAAAGAGAACGGCCACGTCGCATGGCCATTGCTGTTTTGTTTGCAACCGTCGAATGCCGCGCCCGGC
dmAMN  CAACAGGGAAAAGCAAATCAAAAGTGCAGGAGCACGGAAAGAGAACGGCCACGTCGCATGGCCATTGCTGTTTTGTTTGCAACCGTCGAATGCCGCGCCCGGC
       3,721  3,731  3,741  3,751  3,761  3,771  3,781  3,791  3,801  3,811
                       Part of intron 5 (EcoRV to EcoRV)
EVE    ACGACCATGCCCACCAGAGACAAAAGTACGCACGCTTCGAGGGCTGACTCACGGATGGTAAAGCTACGGCTGGAGCACCTTCACCATACGAAAATCCTGCCTC
dmAMN  ACGACCATGCCCACCAGAGACAAAAGTACGCACGCTTCGAGGGCTGACTCACGGATGGTAAAGCTACGGCTGGAGCACCTTCACCATACGAAAATCCTGCCTC
       3,826
EVE    AATCGGAT
dmAMN  AATCGGAT
```

Figure S4. The *regA* locus of dmAMN does not contain any mutations in the promoter or any other regions that likely contain regulatory sequences.
Alignment between wild-type EVE and dmAMN sequences of promoter, intron 3, part of intron 5 between two *Eco*RV restriction sites, and intron 7; base numbers are given relative to *regA* transcription initiation site.

Figure S5

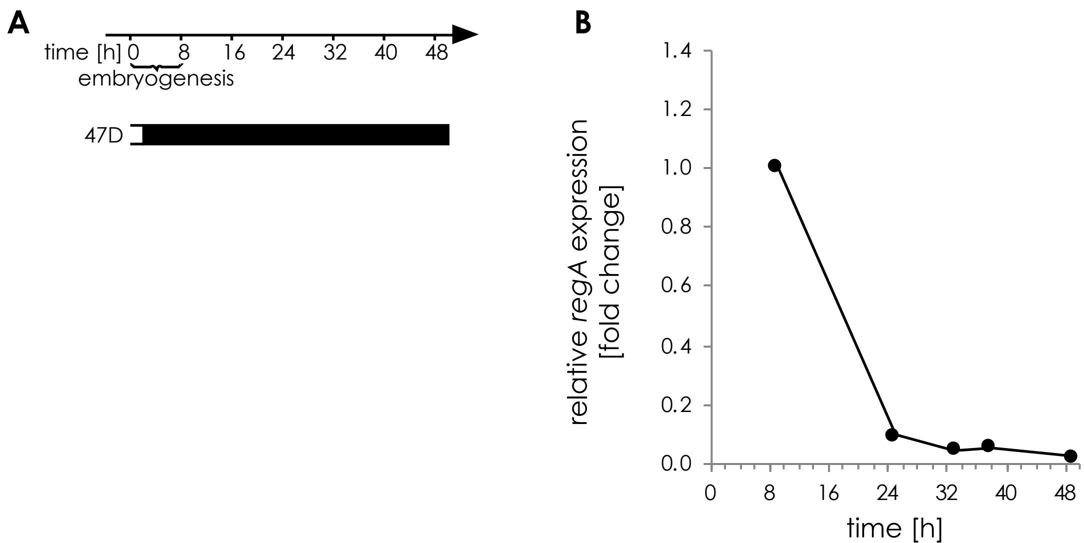

Figure S5. *regA* expression in dmAMN mutant maintained in dark for up to 49 h. (**A**) dmAMN cultures were grown in a standard 16L:8D light regime; under these conditions the onset of embryogenesis (time point 0 h) takes place 2 hours before the end of the light period. Cultures were then maintained in continuous dark and samples were taken at different time points. White and dark bars denote light and dark periods, respectively. (**B**) *regA* transcript levels were measured using RT-qPCR and expression normalized to time point 9 h – which denotes the developmental induction of *regA* at the end of embryogenesis.

Figure S6

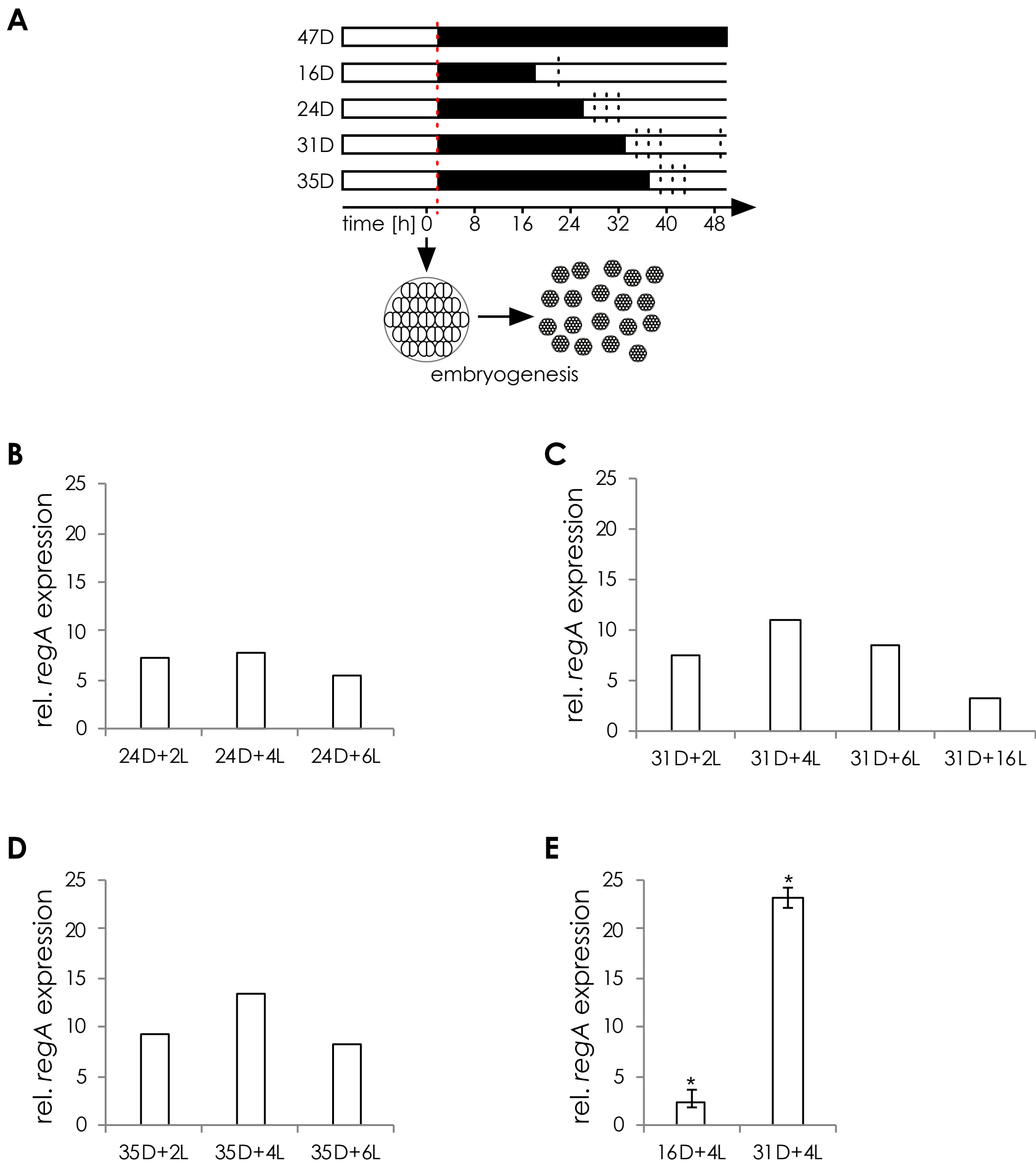

Figure S6. *regA* expression levels are influenced by the duration of both dark and light periods. (**A**) dmAMN cultures were first grown in a standard 16L:8D light regime. After the onset of embryogenesis (time point 0 h) and at the end of the first light period (time point 2 h, red dashed line), cultures were kept in dark, or kept for 16, 24, 31 or 35 h in dark and then exposed to light for 2, 4, 6 or 16 h. White bars, light periods; black bars, dark periods; black dashed lines, time points of collecting samples for RNA extraction; red dashed line, time point 2 h; D, hours of dark; L, hours of light. (**B–E**) *regA* transcript levels in cultures exposed to light relative to dark-maintained cultures from the same time point. (**B–D**) Three independent initial experiments (n = 1) show the relative *regA*

transcript levels after 2, 4, 6, or 16 h of light after (**B**) 24, (**C**) 31, or (**D**) 35 h of dark. (**E**) Comparison between transcript levels at 4 h light exposure after 16 or 31 h of dark ($n = 3$, bars indicate SE; randomization test, $*p < 0.05$).

Figure S7

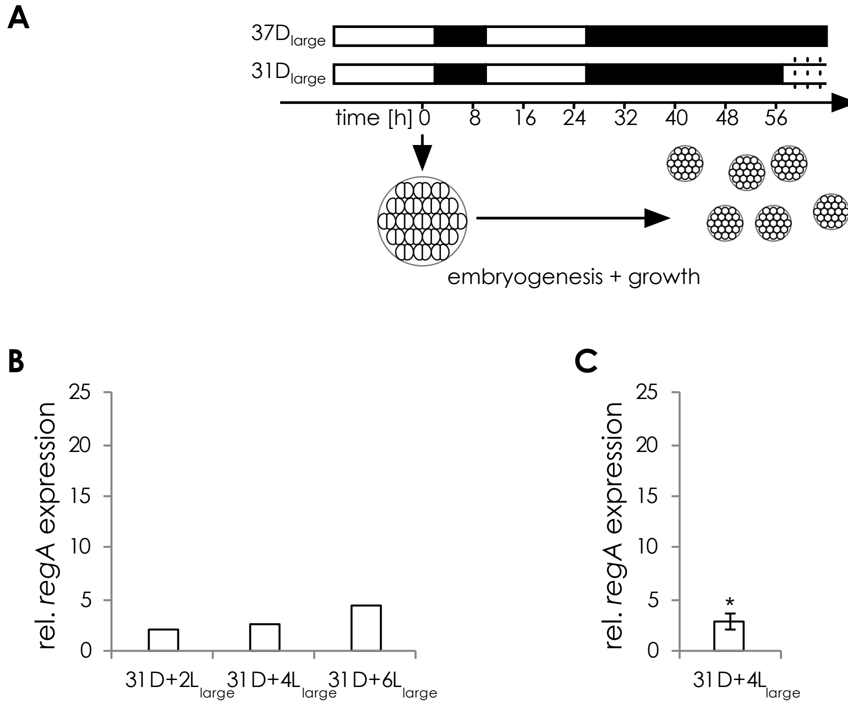

Figure S7. In large dmAMN cells, light only weakly induces *regA* expression. (**A**) dmAMN cultures were first grown in the standard 16L:8D light regime until the end of the first light period after embryogenesis (time point 26 h); during this time cells grew from ca. 4 µm to ca. 8 µm. Cultures were then maintained in dark for 31 h followed by exposure to 2, 4 or 6 h of light ($31D+2L_{large}$, $31D+4L_{large}$, $31D+6L_{large}$) or kept in the dark for the same amount of time (33D, 35D, 37D, respectively). White bars, light periods; black bars, dark periods; dashed lines, time points of samples; D, hours of dark; L, hours of light. (**B–C**) *regA* transcript levels in cultures exposed to light relative to dark-maintained cultures at the same time point. (**B**) In an initial experiment ($n = 1$), cultures were treated for 2, 4, or 6 h with light after 31 h of dark. (**C**) Follow-up experiment in triplicates; cultures exposed to light for 4 h after 31 h of dark ($n = 3$, bars indicate SE; randomization test, $*p < 0.05$).

Figure S8

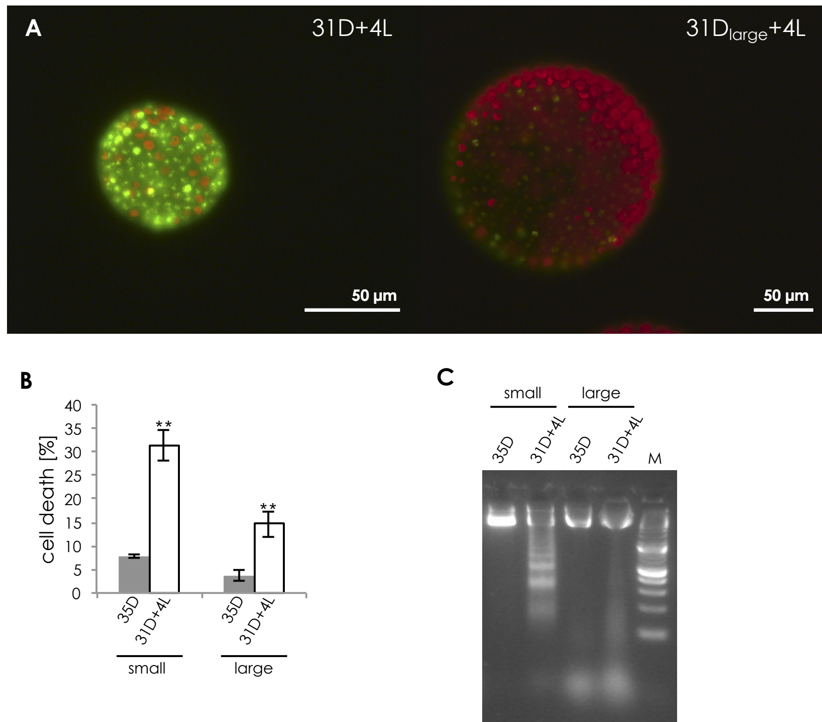

Figure S8. Light causes lower levels of death in large cells relative to small dmAMN cells. (A) After the onset of embryogenesis, dmAMN cultures were either switched to continuous dark at the end of the first light period (small cells), or first grown until the end of the second daylight period (large cells) before being placed in dark for 35 h (35D) or 31 h of dark followed 4 h (31D+4L; light intensity: 260 µmol photons m^{-2} s^{-1}). Green, dead cells (SYTOX Green); red, living cells (chlorophyll autofluorescence).
(B) Comparison between the percentage of dead cells (stained with SYTOX Green) in colonies with small and large cells exposed to 260 µmol photons m^{-2} s^{-1}light intensity (2-sample t-test, $^{**}p < 0.001$). Live and dead cells of one hemisphere of each individual were counted and percent cell death per individual calculated ($n = 3$; 3 technical replicates with ≥ 20 individuals each; bars indicate SE). **(C)** Comparison between DNA laddering effect in small and large dmAMN cells exposed to 4 h light after 31 h of dark (31D+4L). M, DNA marker.

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