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

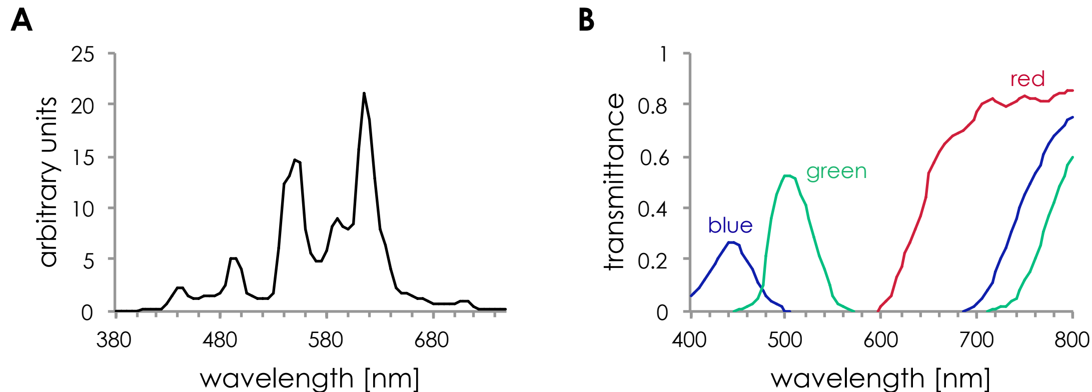

Figure S1 (A) Cool white light spectrum and (B) transmittance of red, green or blue filter (#027, 735, and 071 respectively, LEE Filters).

Figure S2

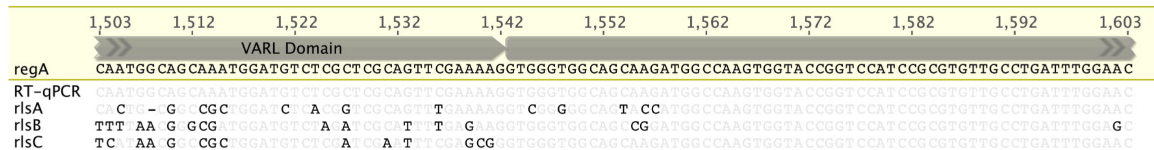

Figure S2. *regA* RT-qPCR specifically amplifies the *regA* locus and not other homologs in the *VARL* gene family.

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

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      -103  -94   -84   -74   -64   -54   -44   -34   -24   -14   -1
                                                Promoter
EVE   ATAAATGCGTACAAGCCAATGCAAGAACGTACATTGTAACTTGTTGTACATTGTCGTTGCGTTGGAGACCCTTCCTAATTGAACTGTTTAAACAATATGGAGC
dmAMN ATAAATGCGTACAAGCCAATGCAAGAACGTACATTGTAACTTGTTGTACATTGTCGTTGCGTTGGAGACCCTTCCTAATTGAACTGTTTAAACAATATGGAGC
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Intron 3

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      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
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Part of Intron 5

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      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

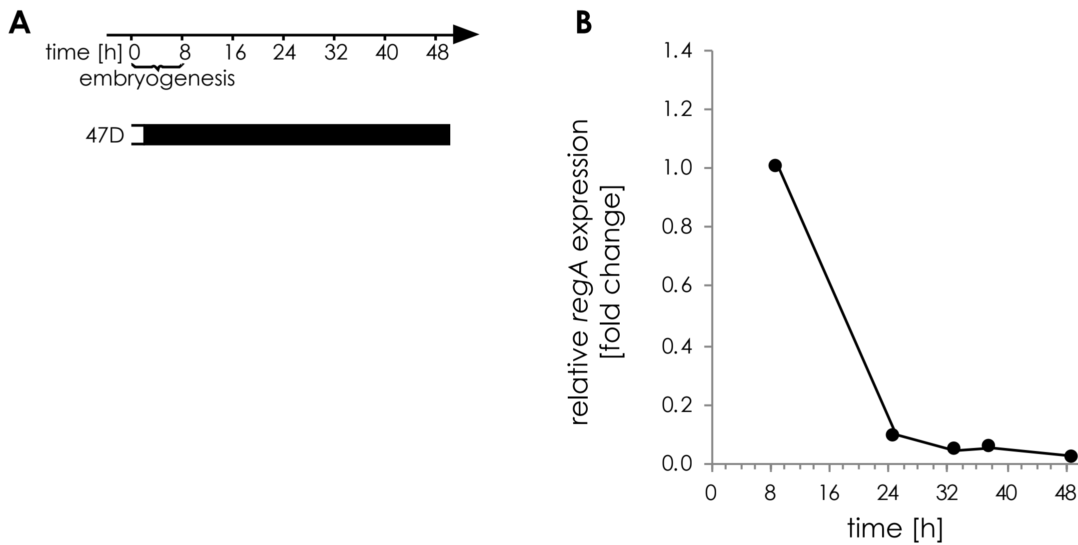

Figure S5. *regA* expression in dmAMN mutant maintained in dark for up to 49 h. (**A**) dmAMN cultures were grown in a standard 16L:8D light regime; under these conditions the onset of embryogenesis (time point 0 h) takes place 2 hours before the end of the light period. Cultures were then maintained 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

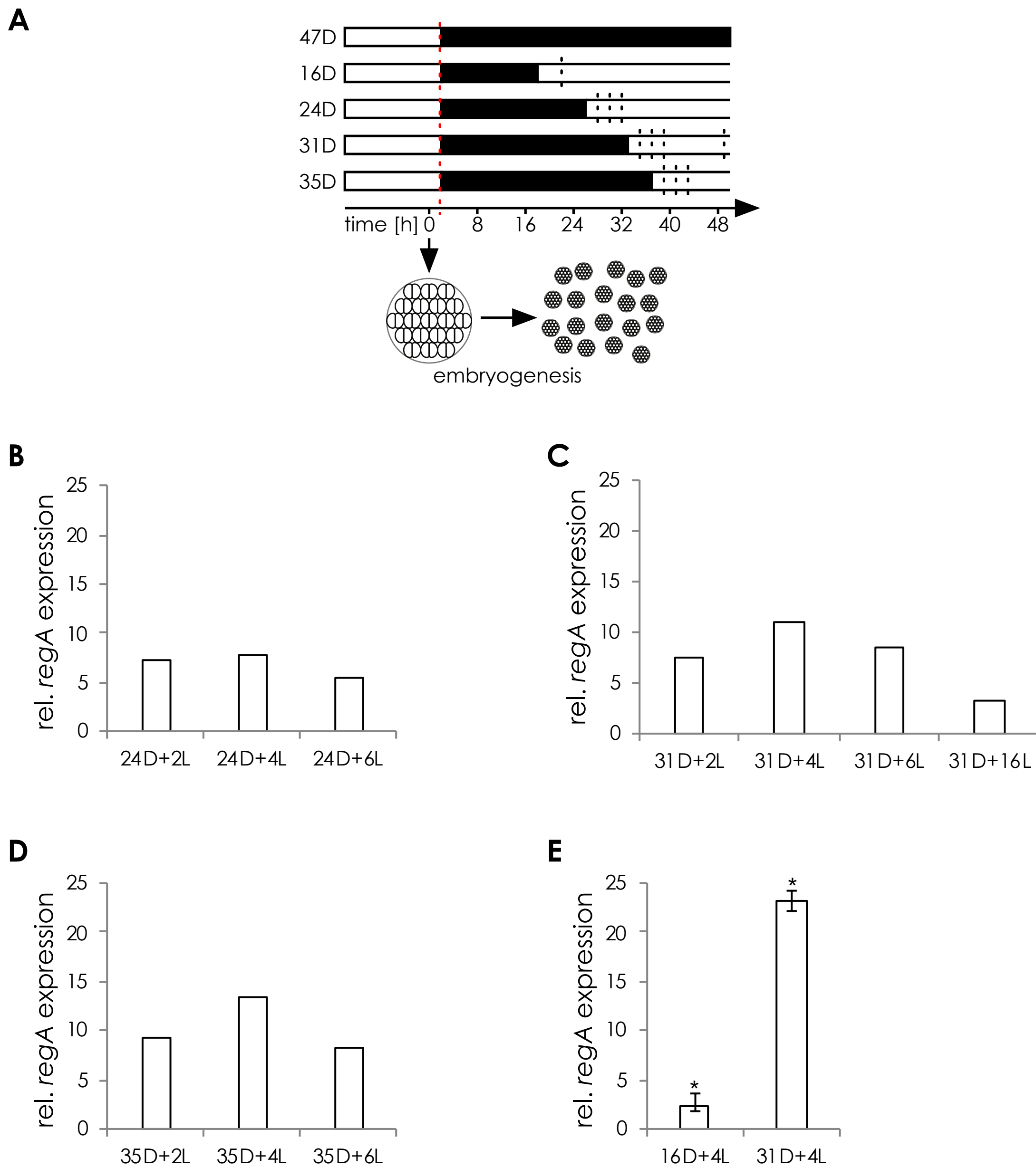

Figure S6. *regA* expression levels are influenced by the duration of both dark and light periods. (**A**) dmAMN cultures were first grown in a standard 16L:8D light regime. After the onset of embryogenesis (time point 0 h) and at the end of the first light period (time point 2 h, red dashed line), cultures 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

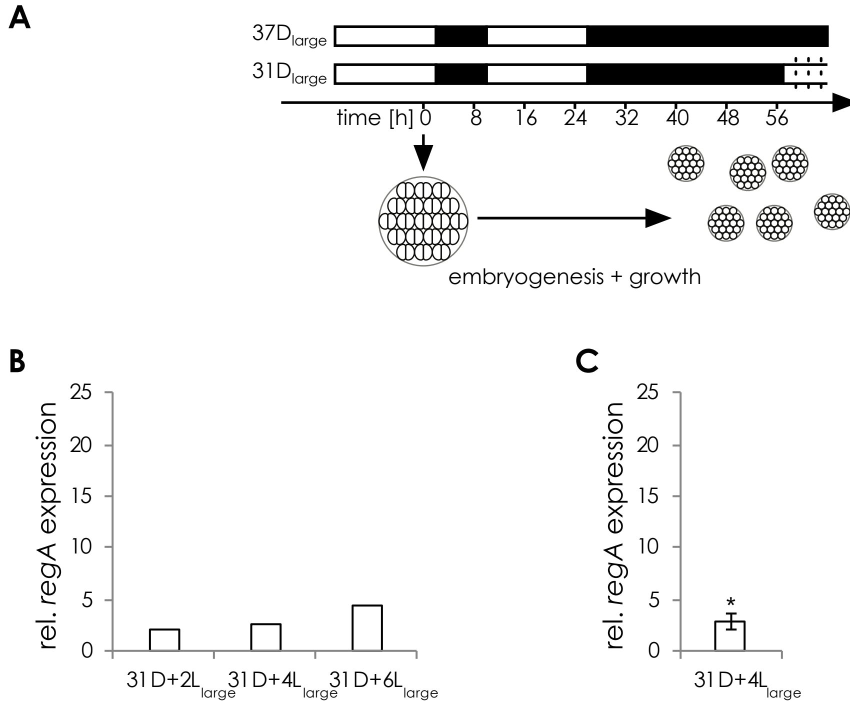

Figure S7. In large dmAMN cells, light only weakly induces *regA* expression.
(**A**) dmAMN cultures were first grown in the standard 16L:8D light regime until the end of the first light period after embryogenesis (time point 26 h); during this time cells grew from ca. 4 µm to ca. 8 µm. Cultures were 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

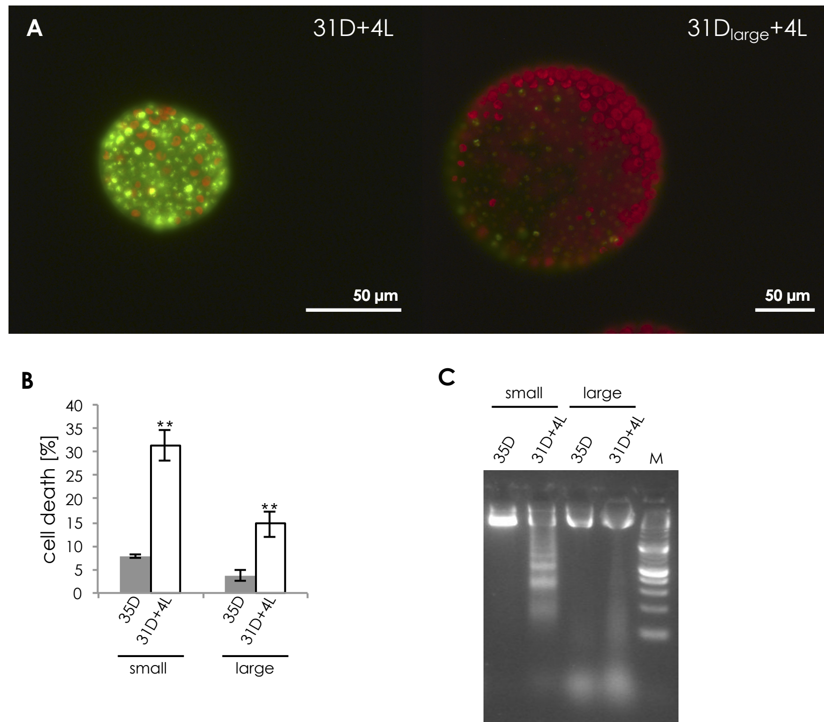

Figure S8. Light causes lower levels of death in large cells relative to small dmAMN cells. (A) After the onset of embryogenesis, dmAMN cultures were either switched to continuous dark at the end of the first light period (small cells), or first grown until the end of the second daylight period (large 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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