SUPPLEMENTARY MATERIAL

Near-future ocean warming and acidification alter foraging behaviour, locomotion, and metabolic rate in a keystone marine mollusc

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Stylocheilus striatus biology

Stylocheilus striatus, as common to most Aplysiidae, has five developmental stages (Fig. S1): (1) embryonic stage (fertilisation to egg hatching) lasting 5-7 days[1,2]; (2) planktonic stage (veligers feed on the phytoplankton) lasting a minimum of 30 days[2]; (3) metamorphic stage (larvae transform into benthic juveniles, which lose the velum and begin grazing on cyanobacteria using a radula) lasting 10-12 days, during which time the parapodia of *Stylocheilus* spp. grow over the shell and the shell is shed when the animal is ~8 mm in length[2]; (4) juvenile stage (after metamorphosis to reproductive maturity) lasting ~17 days[3]; and (5) adult stage. It is important to note that these estimates of developmental stage durations are based on observations of similar Aplysiid species and may differ due to species-specific traits and/or environmental conditions.

Sample collection and experimental treatments

The study was carried out in a seawater flow-through system, in which seawater pH was regulated using a pH controller (IKS Aquastar, Germany) connected to pH electrodes located in 300 L header tanks and calibrated on the National Bureau of Standards (NBS) scale. pH was manipulated by bubbling pure CO_2 into seawater to reach the desired pH level. Temperature in the aquaria was controlled with V2 Therm 200 W digital aquarium heaters (Tropical Marine Centre). Small aquarium pumps (EHEIM compact 300, EHEIM GmbH & Co. KG, Deizisau, Germany) were used in each tank for water circulation.

Behavioural responses setup

The T-maze was made of PVC pipes (4 cm inner diameter) cut in half length-wise and consisted of a starting lane (the stem of the 'T'; 15 cm in length) leading to two choice chambers perpendicular to the starting lane (the arms of the 'T'; each 20 cm in length) that received incoming water at their external ends from two header tanks containing either seawater alone (control cue) or seawater conditioned with *L. majuscula* (stimulus cue). The flow rate into each arm of the T-maze was set to 100 mL min^{-1} using flowmeters. Cyanobacteria (200 g) was added to tanks containing seawater (10 L) 1 h prior to the experiment to condition the seawater. Sea hares were fasted 12 to 24 h before experiments.

This index of speed includes voluntary speed while in motion, but also includes time spent stationary, which was minimal in trials because all individuals were fasted and presumably motivated to feed.

Metabolic rate setup and calculation

The respirometry setup comprised a 40 L (water volume) tank receiving fully aerated flow-through seawater at the target pH and temperature from the 300 L conditioning tanks described above. There were eight 110 mL glass respirometry chambers in which the $\dot{M}_{O_2}$ of the sea hares was measured with the use of oxygen meters and probes (FireStingO2; PyroScience GmbH, Aachen, Germany), a peristaltic pump with gas-tight tubing that recirculated water through the chambers and past the oxygen probes, and a set of flush pumps that intermittently flushed fresh and fully aerated seawater through the respirometry chambers for 3 min in every 12 min intermittent-closed respirometry cycle (i.e. each closed measurement period lasted 9 min). Out of the eight respirometry chambers, at least one was always left empty to monitor

background (microbial) respiration throughout each trial. The respirometry setup was shielded from surrounding disturbances by a large wooden board.

Respiration ($\dot{M}_{O_2}$ in mg O_2 h^{-1}) was calculated by first fitting a linear regression to the data for the decrease in oxygen concentration inside the respirometry chambers over 6.5 min during each of the 9 min closed phases of the respirometry cycles, after which the slopes from these regressions were multiplied by the volume of the respirometry chamber minus sea hare volume (determined by weighing the animals and assuming a density of 1 g mL^{-1}). Since the sea hares did not exhibit elevated $\dot{M}_{O_2}$ after introduction to the respirometry chambers, as is generally seen for vertebrates (e.g. fish) due to the stress from being handled and moved (e.g. Fig. 2 in ref. 4 using the same setup), and also no pronounced flat-line indicative of a standard metabolic rate (e.g. ref. 5), the average (routine) metabolic rate of each individual sea hare was calculated as the mean $\dot{M}_{O_2}$ during the first six hours of each respirometry trial. The first six hours were chosen because background respiration became evident after this period.

Data analysis and statistics

Model structure was the same for each of the three behavioural traits, with either Time to foraging choice, Locomotion speed, or Correct foraging choice as the response variable, Treatment (with nine categories; one control and four temperature/pH treatments for each of the developmentally or adult acclimated groups), Length of sea hares, and Time of day as predictor variables (fixed effects), and Holding tank and Animal ID (since each sea hare was tested twice) as random effects. For Correct foraging choice, we ran a GLME on binomial data (logistic regression) but, since the sea hares always made the correct choice in the control treatment, we randomly assigned one observation as a wrong choice for the control since the logit function is

undefined if the probability is exactly 1. Our analysis of these data is therefore a conservative estimate of the difference between the control treatment and the other treatments (but note that the observed value for the control treatment, i.e. 100% correct foraging choice, is presented graphically in the main article). For the $\dot{M}_{O_2}$ data, the LME had $\log_{10}$-transformed $\dot{M}_{O_2}$ as the response variable, $\log_{10}$-transformed Body mass and Treatment as the predictor variables, and Holding tank as a random effect. For all models, model selection proceeded by dropping variables one by one, starting with the variables with t-values closest to zero. Variables were kept in the models if their inclusion resulted in significantly better fit as indicated by log-likelihood ratio tests. The assumptions of homoscedasticity and normality of residuals were examined by visual inspection of residual-fit plots.

Repeatability (R) was calculated for the behavioural data (as each individual was tested twice) using the *rptR* package[6]. Values presented in the text of the main article are model estimates. For the behavioural data, length of sea hares was never significant in the models (p = 0.375–0.993) and these data are therefore presented graphically (cf. Fig. 3) as their raw (measured) values (i.e. no size-adjustments were performed). For the $\dot{M}_{O_2}$data, on the other hand, body mass was highly significant ($p < 0.0001$) and these data are presented graphically (cf. Fig. 3) as body-mass-adjusted values. Body-mass-adjustments of $\dot{M}_{O_2}$ of individual sea hares were achieved by adding the residuals from the linear regression of $\log_{10}$-transformed $\dot{M}_{O_2}$ *vs.* $\log_{10}$-transformed Body mass across all treatment groups to the $\dot{M}_{O_2}$ predicted from the regression for a 1 g (wet weight) sea hare. The developmentally and adult acclimated groups were combined in the regression since their scaling relationships were similar (developmentally acclimated: intercept = 0.200 mg O_2 h^{-1}, slope (scaling exponent) = 0.733; adult acclimated: intercept = 0.230 mg O_2 h^{-1}, slope = 0.733.

Table S1: Temperature, salinity and carbonate chemistry of seawater. Carbonate chemistry in present-day (pH 8.1) and acidified (pH 7.85 and 7.65) treatments was calculated from pH_{NBS}, total alkalinity (TA), seawater temperature, and salinity using the program CO2SYS[7], selecting the constants from ref. 8. Titration of TA standards were within 1% of that of certified reference material from Dr. A. Dickson (Batch No. 171; Scripps Institution of Oceanography). Mean concentrations, standard deviation (in parentheses) and number of replicates (in italics) are presented for each measurement. DIC = dissolved inorganic carbon, Ω_{arag} = aragonite saturation state.

Treatment	**Temperature (°C)**	**Salinity**	**pH_{NBS}**	**TA (µmol kg^{-1} SW)**	**pCO_2 (µatm)**	**DIC (µmol kg^{-1} SW)**	**HCO_3^- (µmol kg^{-1} SW)**	**CO_3^{2-} (µmol kg^{-1} SW)**	**$CO_{2(aq)}$ (µmol kg^{-1} SW)**	**Ω_{arag}**
pH 8.1 + 28°C (control)	27.85 (0.1) *70*	36.29 (0.05) *35*	8.13 (0.02) *70*	2354.89 (14.48) *12*	466.3 (2.42) *12*	2044.55 (13.07) *12*	1813.21 (11.40) *12*	219.11 (1.53) *12*	12.22 (0.06) *12*	3.48 (0.86) *12*
pH 7.85 + 28°C	27.89 (0.15) *70*	36.28 (0.09) *35*	7.83 (0.03) *70*	2349.65 (24.4) *12*	1015.36 (10.68) *12*	2190.05 (23.32) *12*	2036.35 (21.65) *12*	127.1 (1.36) *12*	26.59 (0.27) *12*	2.02 (0.02) *12*
pH 8.1 + 31°C	30.91 (0.14) *70*	36.26 (0.11) *35*	8.12 (0.02) *70*	2349.17 (13.51) *8*	486.66 (3.8) *8*	2023.55 (11.65) *8*	1782.26 (10.1) *8*	229.4 (2.43) *8*	11.88 (0.1) *8*	3.71 (0.04) *8*
pH 7.85 + 31°C	30.88 (0.14) *70*	36.26 (0.06) *35*	7.86 (0.02) *70*	2355.61 (24.6) *8*	1026.63 (11.02) *8*	2178.1 (23.44) *8*	2014.25 (21.62) *8*	138.75 (1.48) *8*	25.09 (0.26) *8*	2.24 (0.02) *8*
pH 7.65 + 28°C	27.99 (0.1) *70*	36.305 (0.08) *35*	7.65 (0.03) *70*	2340.06 (7.56) *8*	1658.33 (5.48) *8*	2258.31 (7.43) *8*	2129.51 (7) *8*	85.47 (0.28) *8*	43.33 (0.14) *8*	1.36 (0.04) *8*

Table S2: Samples sizes (number of animals) for behavioural and metabolic rate measurements.

Treatment	Developmental acclimation	Adult acclimation
Behaviour		
pH_{NBS} 8.1 + 28°C (control)	15	
pH_{NBS} 7.85 + 28°C	15	10
pH_{NBS} 8.1 + 31°C	15	10
pH_{NBS} 7.85 + 31°C	15	10
pH_{NBS} 7.65 + 28°C	15	10
Metabolic rate		
pH_{NBS} 8.1 + 28°C (control)	13	
pH_{NBS} 7.85 + 28°C	14	6
pH_{NBS} 8.1 + 31°C	4	5
pH_{NBS} 7.85 + 31°C	5	6
pH_{NBS} 7.65 + 28°C	12	7

Table S3: Output of the final statistical models (i.e. excluding variables that did not significantly improve model fit) for the behavioural and physiological traits investigated. Estimates in parentheses are either back-transformed from logits to probabilities for correct foraging choice or from $\log_{10}$ to raw $\dot{M}_{O_2}$ values for metabolic rate. All pH target values for treatments are in the National Bureau of Standards (NBS) scale. [dev. acclim.] = developmental acclimation group, [adult acclim.] = adult acclimation group.

Response variable (bold) and fixed effects	Estimate	SE	df	*t* (LME) *z* (GLME)	*p-value*
Time to foraging choice (LME model)					
(Intercept) pH 8.1 + 28ºC [control]	2.217	0.059	106	37.78	< 0.0001
pH 7.85 + 28ºC [dev. acclim.]	3.689	0.083	106	17.74	< 0.0001
pH 8.1 + 31ºC [dev. acclim.]	5.037	0.083	106	33.99	< 0.0001
pH 7.85 + 31ºC [dev. acclim.]	6.676	0.083	106	53.73	< 0.0001
pH 7.65 + 28ºC [dev. acclim.]	8.492	0.083	106	75.62	< 0.0001
pH 7.85 + 28ºC [adult acclim.]	3.817	0.093	106	17.25	< 0.0001
pH 8.1 + 31ºC [adult acclim.]	5.808	0.093	106	38.70	< 0.0001
pH 7.85 + 31ºC [adult acclim.]	7.378	0.093	106	55.63	< 0.0001
pH 7.65 + 28ºC [adult acclim.]	9.290	0.093	106	76.23	< 0.0001
Correct foraging choice (GLME model)					
(Intercept) pH 8.1 + 28ºC [control]	3.367 (0.967)	1.017		3.311	0.0009
pH 7.85 + 28ºC [dev. acclim.]	1.872 (0.867)	1.150		–1.300	0.1935
pH 8.1 + 31ºC [dev. acclim.]	1.189 (0.767)	1.105		–1.971	0.0487
pH 7.85 + 31ºC [dev. acclim.]	0.546 (0.633)	1.085		–2.599	0.0094
pH 7.65 + 28ºC [dev. acclim.]	–0.134 (0.467)	1.081		–3.239	0.0012
pH 7.85 + 28ºC [adult acclim.]	1.734 (0.850)	1.194		–1.367	0.1716
pH 8.1 + 31ºC [adult acclim.]	0.847 (0.700)	1.128		–2.234	0.0255
pH 7.85 + 31ºC [adult acclim.]	0.405 (0.600)	1.115		–2.657	0.0079
pH 7.65 + 28ºC [adult acclim.]	–0.406 (0.400)	1.115		–3.384	0.0007
Locomotion speed (LME model)					
(Intercept) pH 8.1 + 28ºC [control]	9.695	0.122	106	79.81	< 0.0001
pH 7.85 + 28ºC [dev. acclim.]	6.157	0.172	106	–20.59	< 0.0001
pH 8.1 + 31ºC [dev. acclim.]	4.457	0.172	106	–30.49	< 0.0001
pH 7.85 + 31ºC [dev. acclim.]	3.525	0.172	106	–35.92	< 0.0001
pH 7.65 + 28ºC [dev. acclim.]	2.691	0.172	106	–40.77	< 0.0001
pH 7.85 + 28ºC [adult acclim.]	6.065	0.192	106	–18.90	< 0.0001
pH 8.1 + 31ºC [adult acclim.]	4.031	0.192	106	–29.49	< 0.0001
pH 7.85 + 31ºC [adult acclim.]	3.173	0.192	106	–33.96	< 0.0001
pH 7.65 + 28ºC [adult acclim.]	2.398	0.192	106	–37.99	< 0.0001
Metabolic rate (LME model)					
(Intercept) pH 8.1 + 28ºC [control]	–0.711 (0.194)	0.038		–18.572	< 0.0001
Body mass	0.730	0.109		6.708	< 0.0001
pH 7.85 + 28ºC [dev. acclim.]	–0.777 (0.167)	0.035		–1.898	0.0624
pH 8.1 + 31ºC [dev. acclim.]	–0.666 (0.216)	0.055		0.806	0.4235
pH 7.85 + 31ºC [dev. acclim.]	–0.573 (0.267)	0.047		2.905	0.0051
pH 7.65 + 28ºC [dev. acclim.]	–0.657 (0.220)	0.036		1.500	0.1387
pH 7.85 + 28ºC [adult acclim.]	–0.661 (0.218)	0.045		1.125	0.2648
pH 8.1 + 31ºC [adult acclim.]	–0.670 (0.214)	0.048		0.855	0.3958
pH 7.85 + 31ºC [adult acclim.]	–0.580 (0.263)	0.046		2.841	0.0061
pH 7.65 + 28ºC [adult acclim.]	–0.641 (0.229)	0.046		1.529	0.1313

Table S4: Results of the chi-squared tests (two-sided) for probability of success for sea hares making correct foraging choices. All pH target values for treatments are in the National Bureau of Standards (NBS) scale.

Treatment	**Acclimation type**	χ^2	**df**	***p*-value**
pH 8.1 + 28°C (control)		30	1	<0.0001
pH 7.85 + 28°C	Developmental	16.13	1	<0.0001
pH 7.85 + 28°C	Adult	9.80	1	0.0017
pH 8.1 + 31°C	Developmental	8.53	1	0.0035
pH 8.1 + 31°C	Adult	3.20	1	0.0736
pH 7.85 + 31°C	Developmental	2.13	1	0.1441
pH 7.85 + 31°C	Adult	0.80	1	0.3711
pH 7.65 + 28°C	Developmental	0.13	1	0.7150
pH 7.65 + 28°C	Adult	0.80	1	0.3711

Table S5: Pairwise multiple comparisons of means between developmentally and adult acclimated sea hares within each treatment. All pH target values for treatments are in the National Bureau of Standards (NBS) scale.

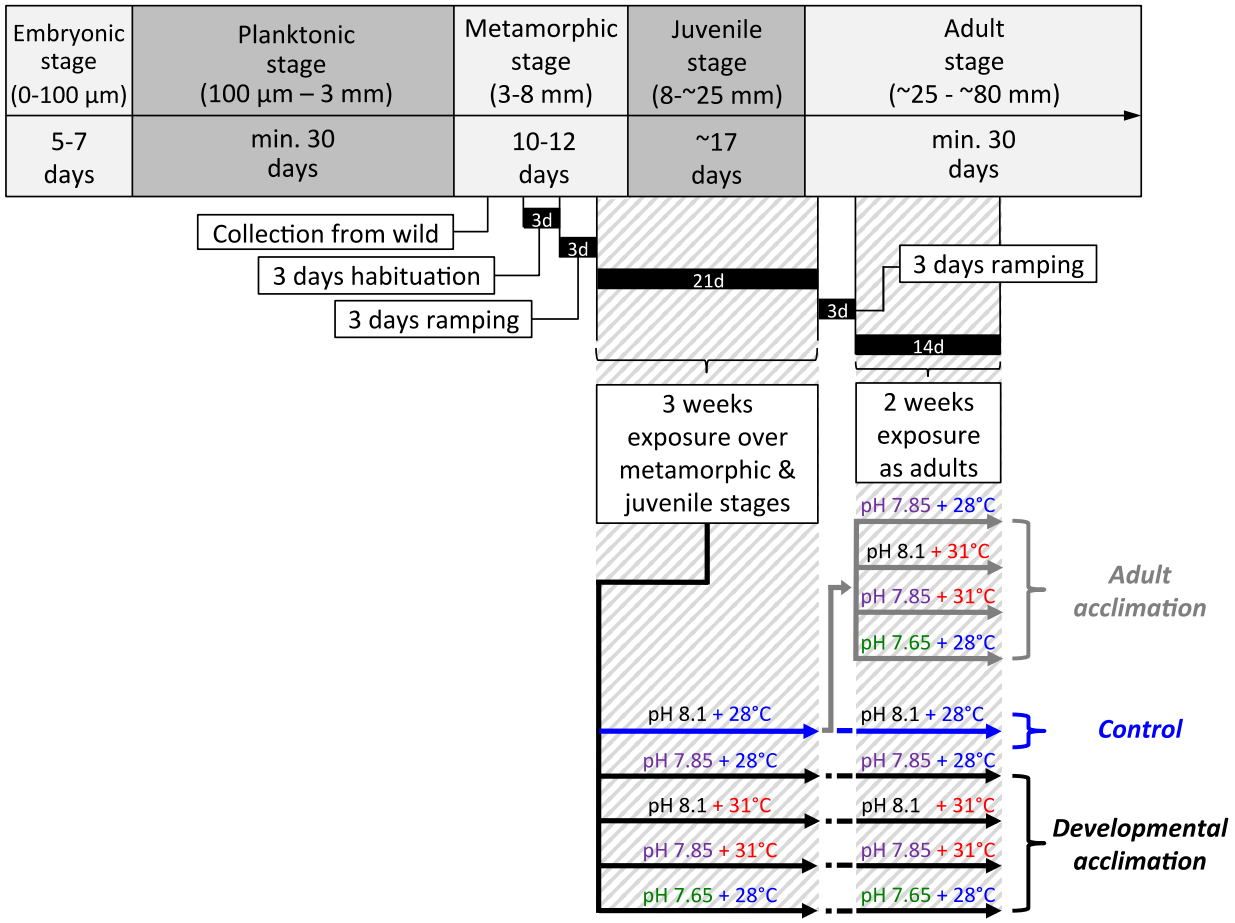
Treatments compared	Estimate	SE	*t (metabolic rate)* *z (all other traits)*	*p-value*
Time to foraging choice				
pH 7.85 + 28ºC	–0.128	0.093	–1.380	0.5200
pH 8.1 + 31ºC	–0.771	0.093	–8.306	< 0.0001
pH 7.85 + 31ºC	–0.702	0.093	–7.566	< 0.0001
pH 7.65 + 28ºC	–0.797	0.093	–8.592	< 0.0001
Correct foraging choice				
pH 7.85 + 28ºC	0.137	0.825	0.166	1.000
pH 8.1 + 31ºC	0.342	0.652	0.525	0.974
pH 7.85 + 31ºC	0.141	0.593	0.238	0.999
pH 7.65 + 28ºC	0.272	0.585	0.465	0.984
Locomotion speed				
pH 7.85 + 28ºC	0.093	0.192	0.483	0.981
pH 8.1 + 31ºC	0.426	0.192	2.220	0.102
pH 7.85 + 31ºC	0.353	0.192	1.837	0.240
pH 7.65 + 28ºC	0.293	0.192	1.527	0.418
Metabolic rate				
pH 7.85 + 28ºC	–0.116	0.044	–2.622	0.0428
pH 8.1 + 31ºC	0.004	0.063	0.064	1.0000
pH 7.85 + 31ºC	0.006	0.056	0.115	0.9999
pH 7.65 + 28ºC	–0.016	0.048	–0.330	0.9953

Figure legends

Figure S1: *Stylocheilus striatus* developmental life stages, photographs and experimental timeline.

(**a**) The five developmental stages of *S. striatus* are shown: (1) embryonic stage (fertilisation to egg hatching); (2) planktonic stage (veligers feed on the phytoplankton); (3) metamorphic stage (larvae transform into benthic juveniles, which lose the velum and begin grazing on cyanobacteria using a radula); (4) juvenile stage (after metamorphosis to reproductive maturity); and (5) adult stage. The experimental timeline is also shown. Sea hares were exposed to their respective treatments for three weeks until reaching their adult stage (i.e. developmental acclimation; shown by diagonal grey stripes. Then, a set of adult sea hares reared in ambient conditions (control; pH 8.1 + 28°C) were transferred to each of the four treatments with modified temperature and/or pH to serve as the adult acclimated group. The two experimental sea hare groups were then kept in their respective seawater treatment for an additional two weeks (shown by diagonal grey stripes). A third group of individuals were maintained under control ambient conditions for the whole period. (**b**) Photographs of the five developmental stages of *S. striatus* are also provided.

189 **(a)**


Embryonic stage (0-100 µm)
Planktonic stage (100 µm – 3 mm)
Metamorphic stage (3-8 mm)
Juvenile stage (8-~25 mm)
Adult stage (~25 - ~80 mm)
5-7 days
min. 30 days
10-12 days
~17 days
min. 30 days
Collection from wild
3 days habituation
3 days ramping
3d
3d
21d
3d
3 days ramping
14d
3 weeks exposure over metamorphic & juvenile stages
2 weeks exposure as adults
pH 7.85 + 28°C
pH 8.1 + 31°C
pH 7.85 + 31°C
pH 7.65 + 28°C
Adult acclimation
pH 8.1 + 28°C
pH 8.1 + 28°C
Control
pH 7.85 + 28°C
pH 7.85 + 28°C
pH 8.1 + 31°C
pH 8.1 + 31°C
pH 7.85 + 31°C
pH 7.85 + 31°C
pH 7.65 + 28°C
pH 7.65 + 28°C
Developmental acclimation

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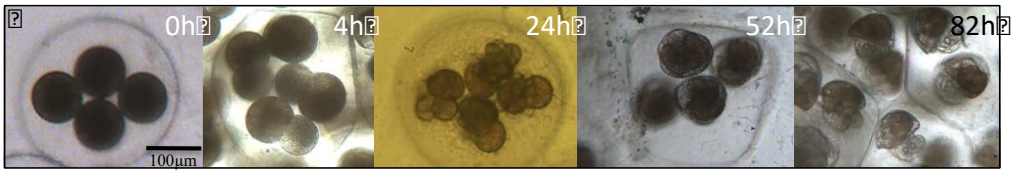
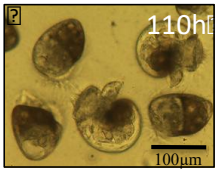
191 **(b)**

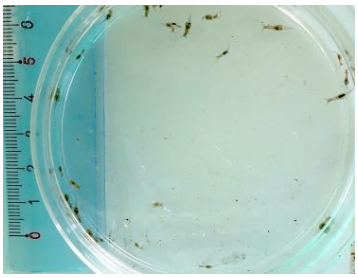
Embryonic stages: First division of cleavage 4h; Four-cell stage 24h; Gastrula 52h; Early veliger stage 82h. Photographs by Suzanne Mills.



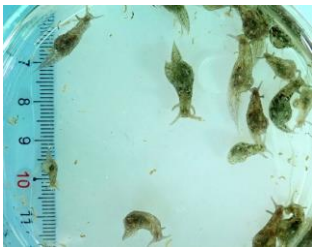
Planktonic stage: Hatched veligers. Photograph by Suzanne Mills.



Metamorphic stage: Photograph by Jennie Pistevos.



Juvenile stage: Photograph by Jennie Pistevos.



Adult stage: Photograph by Suzanne Mills.

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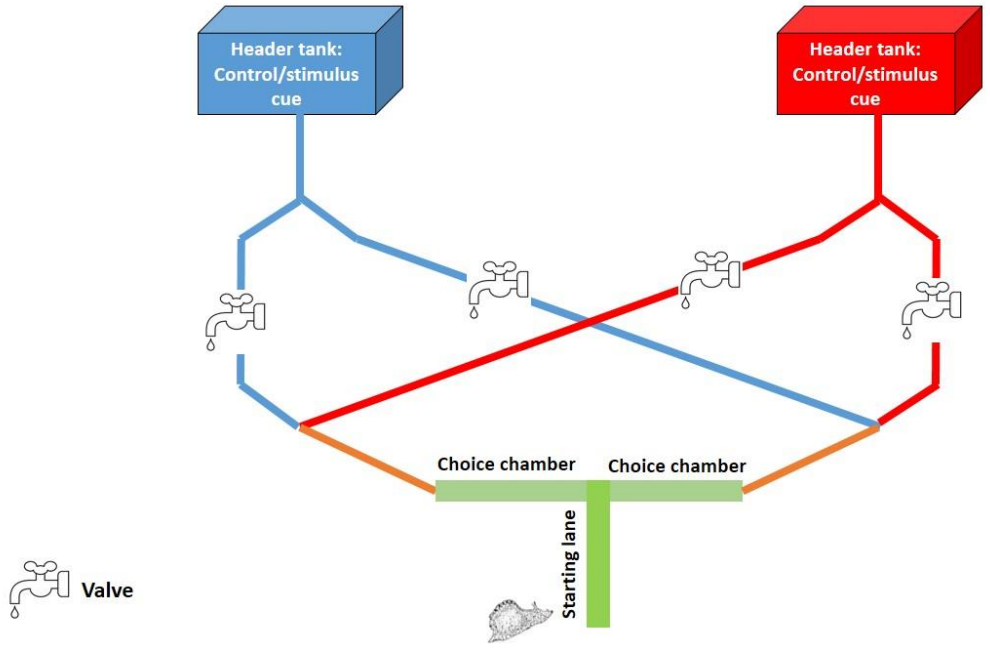
Figure S2: Diagram showing the T-maze setup. The T-maze consisted of a starting lane (the stem of the 'T'; 15 cm in length) leading to two choice chambers perpendicular to the starting lane (the arms of the 'T'; each 20 cm in length) which received incoming water through a series of valves at their external ends from two header tanks containing either seawater alone (control cue) or seawater conditioned with the cyanobacterium *Lyngbya majuscula* (stimulus cue).



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