

## SHORT COMMUNICATION

# Bioluminescence induction in the ophiuroid *Amphiura filiformis* (Echinodermata)

Jerome Mallefet\*, Laurent Duchatelet and Constance Coubris

## ABSTRACT

Bioluminescence is a widespread phenomenon in the marine environment. Among luminous substrates, coelenterazine is the most widespread luciferin, found in eight phyla. The wide phylogenetic coverage of this light-emitting molecule has led to the hypothesis of its dietary acquisition, which has so far been demonstrated in one cnidarian and one lophogastrid shrimp species. Within Ophiuroidea, the dominant class of luminous echinoderms, *Amphiura filiformis* is a model species known to use coelenterazine as substrate of a luciferin/luciferase luminous system. The aim of this study was to perform long-term monitoring of *A. filiformis* luminescent capabilities during captivity. Our results show (i) depletion of luminescent capabilities within 5 months when the ophiuroid was fed a coelenterazine-free diet and (ii) a quick recovery of luminescent capabilities when the ophiuroid was fed coelenterazine-supplemented food. The present work demonstrates for the first time a trophic acquisition of coelenterazine in *A. filiformis* to maintain light emission capabilities.

**KEY WORDS:** Coelenterazine, Luciferase, Trophic acquisition, Brittle star

## INTRODUCTION

Bioluminescence, the capacity of living organisms to emit visible light via a biochemical reaction, is a widespread phenomenon in marine ecosystems. Almost 76% of bioluminescent organisms, from bacteria to fishes, live between the surface and a depth of 4000 m (Herring, 1987; Haddock et al., 2010, 2017; Widder, 2010; Martini and Haddock, 2017; Martini et al., 2019). The luminous reaction implies the oxidation of a luciferin either catalysed by the enzyme luciferase or within a cofactor-based stabilized complex called a photoprotein (Haddock et al., 2010; Shimomura, 2012). Light production is involved in various behaviours such as predation, defence against predation and intraspecific communication (Haddock et al., 2010, 2017; Widder, 2010; Jones and Mallefet, 2013).

To date, only four luciferins have been chemically characterized in the marine environment: aldehydes in bacteria, tetrapyrroles in dinoflagellates and imidazopyrazines initially described in Coelenterata (coelenterazine) and Ostracoda (vargulin) (Campbell and Herring, 1990; Thomson et al., 1997; Haddock et al., 2010; Kaskova et al., 2016). Coelenterazine is a luciferin detected in various marine taxa such as radiolarians, cnidarians, ctenophores,

molluscs, crustaceans, echinoderms, chaetognaths and fishes. This large phylogenetic coverage has led to the hypothesis of dietary acquisition of coelenterazine (Shimomura, 1987, 2012; Mallefet and Shimomura, 1995; Thomson et al., 1997; Duchatelet et al., 2019). It must be pointed out that up to now, only one species, *Metridia pacifica*, has been demonstrated to synthesize coelenterazine *de novo* (Oba et al., 2009).

Trophic acquisition of luciferins has been verified experimentally in four species: a jellyfish (*Aequorea victoria*) and a lophogastrid shrimp (*Gnathophausia ingens*) for coelenterazine and two fishes (*Porichthys notatus* and *Parapriacanthus ransonneti*) for vargulin. These experiments demonstrated bioluminescence induction after an exogenous input of luciferin in individuals that had lost their luminescent capabilities (*A. victoria* and *G. ingens*) or in individuals from a non-luminescent population (*P. notatus*) (Warner and Case, 1980; Frank et al., 1984; Thompson et al., 1988; Haddock et al., 2001). Recently, the bioluminescent fish *P. ransonneti* has been shown to obtain not only its luciferin but also its luciferase from luminescent ostracod prey (Bessho-Uehara et al., 2020).

Bioluminescent species have been described in four of the five classes of echinoderms: Crinoidea, Holothuroidea, Asteroidea and Ophiuroidea. Within these classes, luminous ophiuroids represent more than half of the bioluminescent echinoderms described to date (Mallefet, 2009). Over the last decade, biochemical studies have been conducted on the luminous system of *Amphiura filiformis*, a brittle star found in the North Sea and Mediterranean Sea. This infaunal ophiuroid feeds on suspended organic particles by extending two arms in the water column (Rosenberg and Lundberg, 2004; Delroisse et al., 2017a). *Amphiura filiformis* arms are covered with spines containing light-emitting cells called photocytes (Delroisse et al., 2017a). Previous work has shown that the bioluminescent capabilities in *A. filiformis* depend on (i) coelenterazine (Mallefet, 2009; Delval and Mallefet, 2010) and (ii) a luciferase homologous to the *Renilla reniformis* luciferase (Mallefet, 2009; Shimomura, 2012; Delroisse et al., 2017b).

Given that the luminous system of *A. filiformis* is coelenterazine dependent, it represents a good model to follow *in vivo* luminescence capabilities under controlled conditions to test the dietary acquisition hypothesis for the first time in echinoderms.

## MATERIALS AND METHODS

### Sampling

Individuals of *A. filiformis* (Müller 1776) ( $n=205$ ) were collected with an Eckman grab at a depth of 30–40 m in the Gullmarsfjord near the Sven Lovén Centre marine station (Kristineberg, Sweden) in April 2014. Muddy sediment was carefully rinsed off the ophiuroids and intact specimens were transferred into aquaria containing clean sediment and running seawater pumped directly from the adjacent fjord (12°C, 35 salinity). Individuals of *Amphiura chiajei* Forbes 1843 were collected with the same sampling method. Ophiuroids

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were then transported to the Marine Biology Laboratory at the Université Catholique de Louvain (Belgium) where they were kept in captivity in a closed-circuit marine aquarium filled with artificial recirculating seawater (12°C, 35 salinity, low nitrate, pH 8.2). Photoperiod was kept constant for the duration of the experiment (12 h:12 h light:dark)

### Feeding protocol

#### Animal maintenance

For 15 months, coelenterazine-free liquid food containing suspended fine particles (Liquify Marine, Interpet) was provided once a week to the collected ophiuroids.

To monitor luminous capabilities, coelenterazine and luciferase assays were used in addition to KCl induction of luminescence on 95 individuals. These measurements were made once a month from May to September 2014 and again in June and July 2015.

#### Induction protocol

After 15 months in captivity with coelenterazine-free liquid food, specimens were separated into different aquaria (12°C, 35 salinity, low nitrate, pH 8.2) containing 40 mm diameter PVC rings with two ophiuroids in each.

One group ( $n=12$ ) received one dose of standard pellets (Nutra HP 1.0, Skretting, Mozzecane, Italy), as a control experiment to determine luminescence level without induction (day 0 of induction). A second group of 98 ophiuroids received a single dose of coelenterazine-supplemented pellets (Nutra HP 1.0 with 7.52 ng of coelenterazine per gram of food). Finally, another control was performed with *A. chiajei* ( $n=3$ ), a non-luminous sympatric ophiuroid, which were fed with coelenterazine-supplemented pellets. Luminescence capabilities were tested every 3 days over 1 month after induction of luminescence.

### Measurement of luminous capabilities

#### Dissection

Animals were anaesthetized by immersion in  $\text{MgCl}_2$  solution (3.5%) for 3 min (Dewael and Mallefet, 2002; Dupont and Thorndyke, 2006). For each specimen, three arms were removed from the disc and weighed. One arm was placed in artificial sea water (ASW; 400 mmol  $\text{l}^{-1}$  NaCl, 9.6 mmol  $\text{l}^{-1}$  KCl, 52.3 mmol  $\text{l}^{-1}$   $\text{MgCl}_2$ , 9.9 mmol  $\text{l}^{-1}$   $\text{CaCl}_2$ , 27.7 mmol  $\text{l}^{-1}$   $\text{Na}_2\text{SO}_4$ , 20 mmol  $\text{l}^{-1}$  Tris; pH 8.2) for KCl induction of luminescence (see below), while the other two were frozen rapidly at  $-80^\circ\text{C}$  for biochemical assays.

#### Luminescence measurements

Measurements of light emission were carried out in a dark room using an FB12 tube luminometer (Tirtertek-Berthold, Pforzheim, Germany) calibrated using a standard 470 nm light source (Beta light, Saunders Technology, Hayes, UK). Light responses were recorded using FB12-Sirius PC Software (Tirtertek-Berthold). Light emission was characterized as follows: (i) the maximum light intensity ( $i_{\text{max}}$ ), expressed in quanta per second (quanta  $\text{s}^{-1}$ ), and (ii) the total amount of light emitted ( $L_{\text{tot}}$ ) over 3 min, expressed in quanta. All data were standardized per unit of mass (g).

#### KCl induction of luminescence

Before KCl induction of luminescence, 500  $\mu\text{l}$  of ASW was added to a tube containing one arm. Then, light emission was initiated with the addition of 500  $\mu\text{l}$  of KCl solution (400 mmol  $\text{l}^{-1}$  KCl, 52.3 mmol  $\text{l}^{-1}$   $\text{MgCl}_2$ , 9.9 mmol  $\text{l}^{-1}$   $\text{CaCl}_2$ , 27.7 mmol  $\text{l}^{-1}$   $\text{Na}_2\text{SO}_4$ , 20 mmol  $\text{l}^{-1}$  Tris; pH 8.2).  $L_{\text{tot}}$  was recorded and converted into quanta per gram of arm segment (quanta  $\text{g}^{-1}$ ).

### Coelenterazine and luciferase assays

For coelenterazine detection, one of the frozen ophiuroid arms was put into 200  $\mu\text{l}$  of cold argon-saturated methanol and crushed with mortar and pestle. Then, 5  $\mu\text{l}$  of the methanolic extract was injected into a tube filled with 195  $\mu\text{l}$  of Tris buffer (20 mmol  $\text{l}^{-1}$  Tris, 0.5 mol  $\text{l}^{-1}$  NaCl; pH 7.4) and placed in the luminometer. Afterwards, 200  $\mu\text{l}$  of *Renilla* luciferase solution (3  $\mu\text{l}$  of *Renilla* luciferase, 197  $\mu\text{l}$  of Tris buffer) was injected into the luminometer tube.  $L_{\text{tot}}$  was recorded and used to calculate the amount of coelenterazine contained in a gram of arm tissue (ng  $\text{g}^{-1}$ ) assuming that 1 ng of pure coelenterazine coupled with *Renilla* luciferase emits  $2.52 \times 10^{11}$  photons (Shimomura, 2012).

For the luciferase assay, the other frozen ophiuroid arm was placed in 100  $\mu\text{l}$  of Tris buffer and crushed with mortar and pestle until a homogenized extract was obtained; 20 and 40  $\mu\text{l}$  of the extract was diluted in 180 and 160  $\mu\text{l}$  Tris buffer, respectively. The diluted *A. filiformis* luciferase solutions were injected into two different tubes filled with 5  $\mu\text{l}$  of 1/200 stock solution of coelenterazine (Prolume Ltd, Pinetop, AZ, USA) in cold methanol (1 OD at 430 nm) diluted in 195  $\mu\text{l}$  of Tris buffer. Two measures of maximum light emission were recorded and averaged to calculate the maximal light decay rate corresponding to the luciferase activity, expressed in  $10^9$  quanta  $\text{s}^{-1}$   $\text{g}^{-1}$  (Shimomura, 2012).

### Statistical analysis

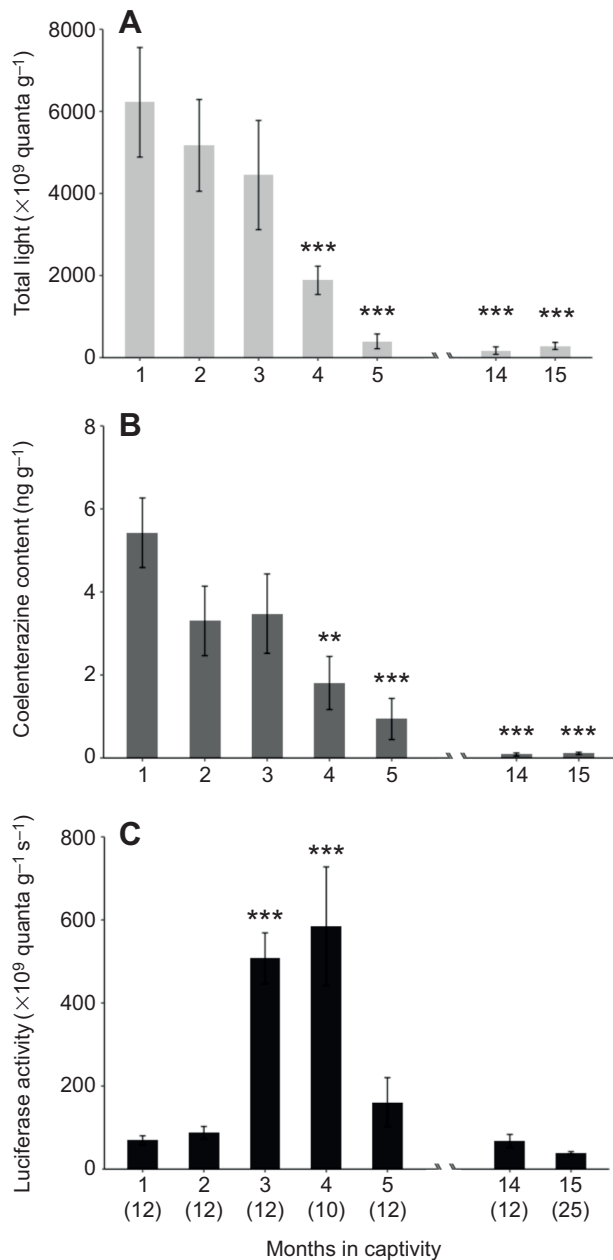
All statistical analyses were performed with R Studio (version 1.1.383, 2009, R Studio Inc., USA). Variance normality and equality were tested by the Shapiro–Wilk test and Levene's test, respectively. For the long-term depletion experiment, these parametric assumptions were not met; therefore, a non-parametric Kruskal–Wallis ANOVA was used to assess the significant difference between more than two groups. All of the pairwise comparisons were tested using a Wilcoxon test to compare groups with the first month. For induction experiments, log and square-root transformations were used to reach normality and homoscedasticity and perform a one-way ANOVA coupled with a Dunnett's multiple comparison test using the day 0 value as the control. When normality and homoscedasticity were not achieved, a non-parametric Kruskal–Wallis ANOVA followed by Wilcoxon multiple comparisons test was used.

Each difference was considered to be significant at minimum  $P < 0.05$ . Values were graphically illustrated with mean and s.e.m.

### RESULTS AND DISCUSSION

Coelenterazine is the substrate of the luminescence reaction found in eight different marine phyla: Radiolaria, Cnidaria, Ctenophora, Mollusca, Arthropoda, Chaetognath, Echinodermata and Chordata. This widespread occurrence of coelenterazine in phylogenetically distant organisms is the main argument in favour of the dietary acquisition hypothesis (Shimomura, 1987, 2012; Mallefet and Shimomura, 1995; Haddock et al., 2010; Widder, 2010).

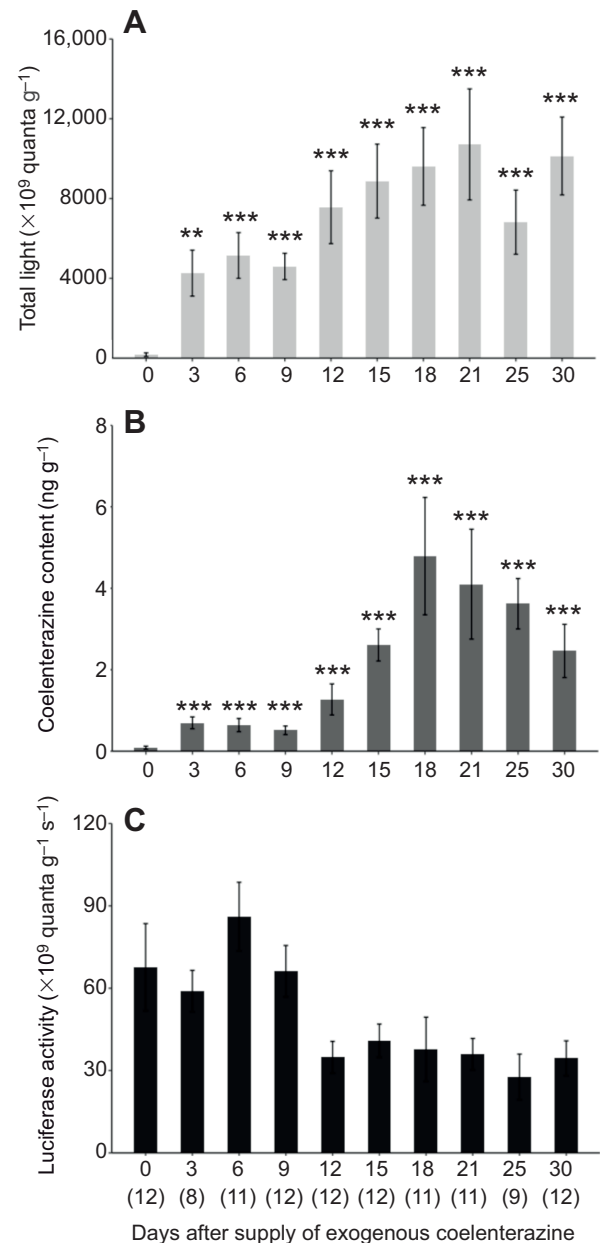
Our results add strong new support to this hypothesis by monitoring for the first time *A. filiformis* luminescence over 16 months in controlled conditions. During the first 15 months, animals were fed with coelenterazine-free liquid food. Statistical analyses revealed that light emission induced by KCl decreased from the first to the fifteenth month of captivity ( $\chi^2=59.6$ ;  $P < 0.001$ ;  $n=95$ ). A significant decline in light emissions appeared after the fourth month of captivity (Fig. 1A; Table S1A). The coelenterazine assay revealed a drop of coelenterazine content between the first and the fifteenth month ( $\chi^2=65.67$ ;  $P < 0.001$ ;  $n=95$ ). A significant decrease of coelenterazine concentration was observed from the



**Fig. 1. Luminescence capabilities of *Amphiura filiformis* in captivity fed coelenterazine-free food.** (A) Total light emission monitored during 15 months of captivity. KCl-induced luminescence decreased significantly after 4 months. (B) Coelenterazine content measured in ophiroid arm tissues during 15 months of captivity. Coelenterazine concentration dropped significantly after 5 months. (C) Luciferase activity quantified during 15 months of captivity. Enzyme activity increased significantly in the third and fourth months. Values are expressed as means  $\pm$  s.e.m. Asterisks indicate a statistical difference from values for the first month of captivity (Kruskal–Wallis and Wilcoxon test; \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). Number of replicates for each month is indicated in parentheses.

fourth month of captivity (Fig. 1B; Table S1A). These results are in agreement with a previous study in which one lophogastrid shrimp (*G. ingens*), from the southern coast of California, showed a loss of its natural luminescence after 4 months in captivity on a coelenterazine-free diet (Frank et al., 1984).

Furthermore, analysis of luciferase assay results revealed that there was a significant variation of luciferase activity during



**Fig. 2. Luminescence capabilities of *A. filiformis* with exogenous coelenterazine.** (A) Total light emission monitored after feeding with coelenterazine-supplemented pellets. KCl-induced luminescence increased significantly 3 days after coelenterazine supplementation. (B) Coelenterazine content monitored after supply of exogenous coelenterazine. Coelenterazine concentration increased significantly 15 days after coelenterazine supplementation. (C) Luciferase activity quantified after feeding with coelenterazine-supplemented pellets. Values are expressed as means  $\pm$  s.e.m. Asterisks indicate a statistical difference from the day 0 (control) value with (i) one-way ANOVA and Dunnett's multiple comparisons test for total light and coelenterazine content, (ii) Kruskal–Wallis ANOVA and Wilcoxon multiple comparisons test for luciferase activity (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). Number of replicates for each time interval is indicated in parentheses.

captivity ( $\chi^2 = 59.01$ ;  $P < 0.001$ ;  $n = 95$ ); an increase of luciferase activity was detected during the third and fourth month of captivity (Fig. 1C; Table S1A), corresponding to July and August. This type of seasonal variation has previously been observed in *Amphipholis squamata* and might be linked to the ophiroid's reproductive cycle (Deheyn et al., 2000). A second peak of luciferase activity would,

therefore, be expected for the fifteenth month. Nevertheless, we hypothesize that the absence of the latter could be explained by the loss of the seasonal cycle regulation due to the lack of photoperiod changes.

To determine whether luminescence disappearance during captivity was due to a lack of coelenterazine in the organism's diet, dietary induction was tested in previously depleted ophiuroids. The results showed that KCl-induced luminescence increased significantly 3 days after the addition of exogenous coelenterazine ( $P < 0.001$ ;  $n = 8$ ). Maximum light emission was reached 21 days after feeding with coelenterazine-supplemented food (Fig. 2A; Table S1B). An increase of coelenterazine content was statistically detected in the ophiuroid arms 3 days after exogenous supply of coelenterazine ( $P < 0.001$ ;  $n = 12$ ). The maximum value was observed 18 days post-supply (Fig. 2B; Table S1B). The results indicated a significant variation of luciferase activity after coelenterazine was provided ( $\chi^2 = 32.64$ ;  $P < 0.001$ ;  $n = 110$ ; Fig. 2C).

According to the induction experiment results, the reacquisition of light emission capability occurred over two steps (days 3–9 and 12–30; Table S2A,B). Comparisons between the first month of depletion and these two plateaus were therefore performed. The results indicate that luminescence and luciferase activity of the first plateau (days 3–9) were not significantly different from those of the first month (Table S2A,B). Conversely, luminescence, coelenterazine content and luciferase activity of the second plateau (days 12–30) were significantly different from those of the first plateau and the first month of captivity (Table S2A,B). Therefore, a two-step recovery occurred, with a first plateau from 3 to 9 days followed by a second plateau from 12 to 30 days, reaching a luminescence level similar to that measured at the beginning of captivity.

Similar (but faster) results have been observed by Frank et al. (1984) in *G. ingens* and by Haddock et al. (2001) in *A. victoria*. In both cases, the ingestion of luminous prey containing coelenterazine induced a reacquisition of luminescence capabilities. The lophogastrid species (*G. ingens*) was able to emit light 2 days after being fed with various luminous prey species (e.g. *Triphoturus mexicanus*, *Gaussia princeps*, *Sergestes similis*; Frank et al., 1984) whereas the hydrozoa (*A. victoria*) reared with coelenterazine-free diet recovered its luminescence capabilities 8 h after ingestion of bioluminescent ctenophores (Haddock et al., 2001). Nevertheless, to date, natural sources of coelenterazine in *A. filiformis* diet remain unknown. Work is in progress to identify organisms containing coelenterazine that are eaten by the ophiuroid.

Tests of luminescence induction failed to trigger any luminous capabilities via coelenterazine supply through the diet on a non-luminous sympatric ophiuroid belonging to the *Amphiura* genus, *A. chiajei*. This ophiuroid did not produce light after being provided with exogenous coelenterazine (Table S2C). This result demonstrates that coelenterazine alone is not sufficient to induce light emission in a closely related, natively non-luminescent organism. In this regard, the bioluminescence reaction is conditioned not only by the presence of luciferin but also by catalytic activity of either a luciferase or a photoprotein (Shimomura and Johnson, 1966; Haddock et al., 2010). As demonstrated in the research on the hydrozoan *A. victoria*, expression of the apophotoprotein gene is uninterrupted, despite the inability to produce light due to the lack of coelenterazine in its diet (Haddock et al., 2001).

Based on our results, it is assumed that coelenterazine is ingested and absorbed before being transferred to the photocytes where luciferase has been detected (Delroisse et al., 2017a). The transfer

process remains unclear but some studies suggested that storage forms (enol-sulfate, dehydrocoelenterazine) of coelenterazine might be involved (Shimomura, 2012). In myctophid fishes, which are known to use coelenterazine as a luminous substrate, storage forms of this luciferin have been detected in the liver and in the digestive tract, suggesting that the more stable storage forms could be stored for future bioluminescence activity (Mallefet and Shimomura, 1995; Duchatelet et al., 2019). The study of these storage forms could provide information about the use of coelenterazine in *A. filiformis* from the moment of its ingestion to light emission in photocytes. In addition, the transfer of the coelenterazine molecule from ingestion to its arrival in the light-emitting cells should be investigated.

In conclusion, the loss of luminous capabilities in *A. filiformis* when exposed to a coelenterazine-free diet is reversible and supports the hypothesis of coelenterazine acquisition through the food chain in one ophiuroid species.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: J.M.; Methodology: J.M.; Software: C.C.; Formal analysis: J.M., L.D., C.C.; Resources: J.M.; Data curation: J.M., L.D., C.C.; Writing - original draft: J.M., L.D., C.C.; Writing - review & editing: J.M., L.D., C.C.; Visualization: C.C.; Supervision: J.M.; Project administration: J.M.; Funding acquisition: J.M.

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#### Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.218719.supplemental>

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