

## Anemonefish oxygenate their anemone hosts at night

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Fig. 3 included some additional text. The correct figure is displayed below.

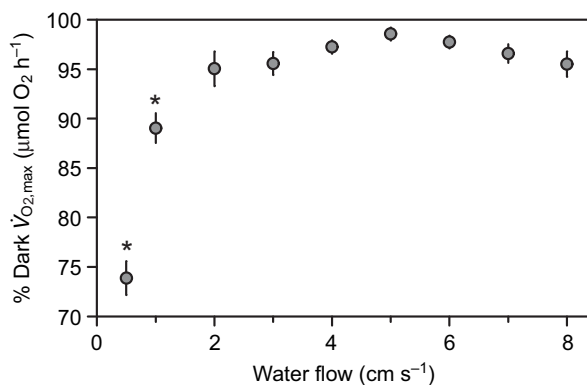


Fig. 3. Effects of water flow rate on the dark maximum oxygen uptake ( $\dot{V}_{O_{2,max}}$ , mean  $\pm$  1 s.e.m.) of bulb-tentacle sea anemones (*E. quadricolor*) in flow-through respirometry. Mean  $\dot{V}_{O_{2,max}}=114.21\pm 13.89 \mu\text{mol O}_2 \text{ h}^{-1}$ .  $\dot{V}_{O_{2,max}}$  values at 0.5 and 1.0  $\text{cm s}^{-1}$  were significantly different (\* $P<0.05$ ).

We apologise to the authors and readers for any confusion that this error may have caused.

## RESEARCH ARTICLE

### Anemonefish oxygenate their anemone hosts at night

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#### SUMMARY

Many stony coral-dwelling fishes exhibit adaptations to deal with hypoxia among the branches of their hosts; however, no information exists on the respiratory ecophysiology of obligate fish associates of non-coral organisms such as sea anemones and sponges. This study investigated metabolic and behavioral interactions between two-band anemonefish (*Amphiprion bicinctus*) and bulb-tentacle sea anemones (*Entacmaea quadricolor*) at night. We measured the net dark oxygen uptake ( $\dot{V}_{O_2}$ ,  $\mu\text{mol O}_2\text{h}^{-1}$ ) of fish–anemone pairs when partners were separate from each other, together as a unit, and together as a unit but separated by a mesh screen that prevented physical contact. We also measured the effects of water current on sea anemone  $\dot{V}_{O_2}$  and quantified the nocturnal behaviors of fish in the absence and presence of host anemones in order to discern the impacts of anemone presence on fish behavior. Net  $\dot{V}_{O_2}$  of united pairs was significantly higher than that of both separated pairs and united pairs that were separated by a mesh screen. Anemone  $\dot{V}_{O_2}$  increased with flow rate from 0.5 to 2.0  $\text{cm s}^{-1}$ , after which  $\dot{V}_{O_2}$  remained constant up to a water flow rate of 8.0  $\text{cm s}^{-1}$ . Furthermore, the percentage time and bout frequency of flow-modulating behaviors by fish increased significantly when anemones were present. We conclude that physical contact between anemonefish and sea anemones elevates the  $\dot{V}_{O_2}$  of at least one of the partners at night, and anemonefish behavior at night appears to oxygenate sea anemone hosts and to augment the metabolism of both partners.

Supplementary material available online at <http://jeb.biologists.org/cgi/content/full/216/6/970/DC1>

Key words: mutualism, ecophysiology, *Amphiprion bicinctus*, sea anemone, oxygen consumption, nocturnal behavior.

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#### INTRODUCTION

Coral reefs are biologically rich and productive ecosystems, second only to tropical rainforests in the diversity of species they harbor (Reaka-Kudla, 1997). Complex mutualistic associations among reef organisms underlie much of this biodiversity (Davies, 1992; Bruno et al., 2003). Many planktivorous coral reef fishes, such as damselfishes, form obligate symbioses with stony corals and sea anemones, leading to the co-evolution and high species diversity of both groups (Fautin and Allen, 1997; Schmitt and Holbrook, 2003; Pinnegar and Polunin, 2006). These sedentary cnidarians cannot move among habitats, and both the hosts and their associated guests are more susceptible to localized environmental stressors than are free-ranging reef organisms.

Many coral associates employ unique ecophysiological adaptations to maintain the benefits of their symbiotic associations amid the weak water flow and hypoxic conditions that can develop among coral branches at night (Goldshmid et al., 2004). Coral-dwelling gobies (Gobiidae, Scorpaenidae) tolerate substantial hypoxia and respire cutaneously to maintain their residence among coral hosts at night (Nilsson et al., 2004; Nilsson and Ostlund-Nilsson, 2004; Nilsson et al., 2007b). Similarly, coral-dwelling damselfishes (Pomacentridae) actively aerate their hosts at night by beating their fins at stroke frequencies two times faster than those during diurnal swimming. This modulation of the hydrodynamic conditions among the coral branches restores nocturnal oxygen availability to that of the ambient water (Goldshmid et al., 2004). Similar metabolic and behavioral adaptations likely are important in mutualisms among fishes and other

types of sessile hosts on coral reefs, such as sponges and sea anemones, but little is known about the ecophysiology of reef symbioses other than those with stony corals.

The mutualism between giant sea anemones and their anemonefish associates on Indo-Pacific coral reefs is one of the most conspicuous and well-known symbioses in marine ecosystems. Initially, the partnership was considered solely as a protection mutualism, in which anemonefish chase away butterfly fishes (Chaetodontidae) that prey on sea anemone tentacles (Fautin, 1991; Fautin and Allen, 1997; Porat and Chadwick-Furman, 2004), and the nematocyst-laden tentacles of sea anemones deter the piscivorous predators of anemonefishes (Mariscal, 1970a). However, recent studies indicate that byproducts from anemonefish wastes provide essential nutrients to their cnidarian hosts (Roopin and Chadwick, 2009; Cleveland et al., 2011; Roopin et al., 2011).

Despite over a century of research on the beneficial interactions between sea anemones and anemonefishes (Collingwood, 1868), nocturnal benefits to the partners, as well as the underlying biological processes involved, remain largely unexplored. This is particularly surprising given the intimate nature of this association at night. During the day, anemonefish spend most of their time in the water column above the anemone, feeding on zooplankton, but each night they nestle deeply among the host tentacles for rest and protection (Allen, 1975; Fautin and Allen, 1997). Investigations of similar cnidarian–fish mutualisms have revealed both metabolic and behavioral adaptations by fish partners in response to variable nocturnal conditions surrounding the sessile hosts (see above), and

thus the close nocturnal association between anemonefishes and sea anemones likely presents major physiological challenges (Goldshmid et al., 2004; Nilsson et al., 2004; Nilsson and Ostlund-Nilsson, 2004; Nilsson et al., 2007b).

Giant sea anemones, like other sessile marine invertebrates on coral reefs, are largely unable to self-modulate the bulk flow of seawater across their tissues, and thus rely on ambient water flow for the mass transfer of essential gases and nutrients (Sebens, 1987). For several decades, researchers have speculated that anemonefishes modulate the flow regime surrounding host sea anemones (Mariscal, 1970a; Allen, 1975; Fautin, 1991; Porat and Chadwick-Furman, 2004; Porat and Chadwick-Furman, 2005), but no quantitative studies have tested this hypothesis. Qualitative observations of *in situ* anemonefish behavior at night indicate that they may be generally inactive (Allen, 1975), adding to speculation about whether these fish modulate gas exchange among host tentacles. However, the highly variable oxygen concentrations and water flow rates on coral reefs, coupled with the tendency of many host anemones to occupy cryptic and topographically complex microhabitats (Sebens, 1997), suggest the potential importance of metabolic and behavioral interactions between sea anemones and anemonefishes at night.

In the present study, we examined nocturnal interactions between bulb-tentacle sea anemones [*Entacmaea quadricolor* (Rüppell and Leuckart 1828)] and two-band anemonefish (*Amphiprion bicinctus*, Rüppell 1830). We tested the hypothesis that this partnership affects the metabolism (oxygen uptake) of the symbiotic partners at night, and specifically that fish-induced water motion affects gas exchange by the sea anemone hosts.

## MATERIALS AND METHODS

### Animal collection and maintenance

In June 2010, wild two-band anemonefish (7–11 cm fork length, FL) and bulb-tentacle sea anemones (11–16 cm tentacle crown diameter, TCD) were collected from shallow coral reefs in the northern Red Sea, adjacent to the Marine Science Station in Aqaba, Jordan (29°27.250'N, 34°58.359'E). Fish and anemones were maintained at the Marine Science Station in flow-through aquaria (80 l) supplied with circulating seawater pumped from the Red Sea. Aquaria were kept on a 12h:12h light:dark photoperiod using halogen lighting (Aqua-Medic, Fort Collins, CO, USA). Fish were fed Formula One Marine Flakes (Ocean Nutrition, San Diego, CA, USA) daily, and anemones were hand-fed frozen fish (*Atherinomorus* spp.) weekly. All experiments on wild animals were conducted within 5–10 days of collection.

At Auburn University, USA, cultured two-band anemonefish were obtained from Oceans, Reefs and Aquariums (Fort Pierce, FL, USA), and bulb-tentacle sea anemones from SunPet (Atlanta, GA, USA) and the New England Aquarium (Boston, MA, USA). Cultured fish and anemones were maintained in 150 l glass aquaria (two fish, one anemone per tank) supplied with artificial seawater and kept on a 12h:12h light:dark photoperiod using high-output fluorescent lighting (Sunlight Supply, Pompano, FL, USA). Fish were fed daily on a mixed diet of Formula One Marine Pellets (Ocean Nutrition, San Diego, CA, USA) and frozen foods (Mysid Shrimp and Emerald Entrée, San Francisco Bay Brand, Newark, CA, USA), and anemones were hand-fed raw shrimp weekly. Experiments on cultured animals (fish 10–12 cm FL, anemones 10–18 cm TCD) were conducted during 2010–2011, after  $\geq 2$  years (fish) or 4–5 weeks (anemones) of maintenance in laboratory aquaria. All wild and cultured animals appeared to be in good physiological condition prior to and during experimental use.

### Patterns of nocturnal oxygen uptake

Metabolic effects of the symbiotic association between fish and anemones were assessed at both the Marine Science Station and Auburn University using flow-through respirometry. Animals were transferred in glass beakers from holding aquaria to cylindrical acrylic chambers (2.5–3.5 l) connected to a recirculating seawater reservoir (150 l, flow rate  $1.0 \pm 0.1 \text{ cm s}^{-1}$ ). Two Clark-type  $\text{O}_2$  electrodes (Strathkelvin Instruments, Motherwell, North Lanarkshire, UK) were attached to the incurrent and excurrent ports of each chamber. To ensure uniform  $\text{O}_2$  availability within the chamber, a stir bar covered by a small mesh cage was positioned in the bottom of the chamber to gently mix the seawater. Oxygen electrodes were calibrated and a 'blank' run was completed prior to each experiment to check for background  $\text{O}_2$  uptake and electrode drift.

Animals were starved for  $\geq 24$  h prior to experimental use, and all experiments were conducted in complete darkness by wrapping the chambers with darkroom curtains. Dark conditions simulated night-time, when anemonefishes reside among sea anemone tentacles for rest and protection (Allen, 1975). Animals were allowed to acclimate in the chambers until a stable, resting respiratory rate was reached (3–6 h, determined visually based on stable  $\dot{V}\text{O}_2$  values on the read-out from the  $\text{O}_2$  meter, Fig. 1). At that point, 20 min of dark  $\text{O}_2$  uptake ( $\dot{V}\text{O}_2$ ,  $\mu\text{mol O}_2 \text{ h}^{-1}$ ) was measured (10 measurements  $\text{min}^{-1} \times 20 \text{ min} = 200$  measurements in total per 20 min treatment) using a two-channel  $\text{O}_2$  meter (Strathkelvin Instruments). Anemones sometimes retracted during the transfer from the holding aquarium to the chamber. The experiment was not initiated if the anemone failed to re-expand to its pre-transfer size, which was measured (TCD, cm) prior to the transfer to the chamber and again before the experiment was initiated. Furthermore, the experiment was not initiated if the anemone did not attach to the bottom of the chamber (e.g. anemone wandered around the chamber).

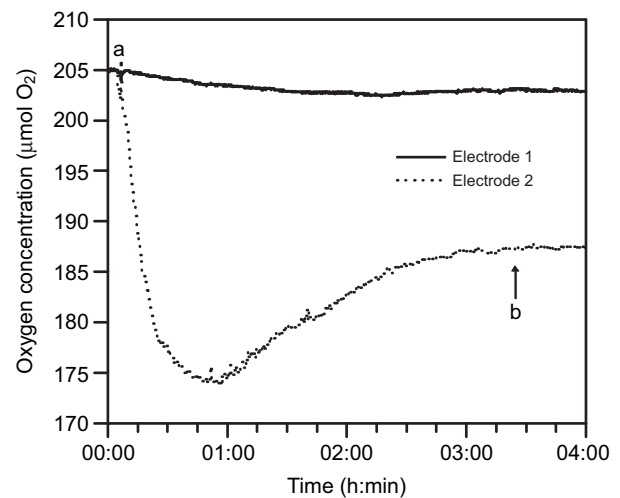


Fig. 1. Representative oxygen meter plot during flow-through respirometry (together treatment) on a two-band anemonefish (*Amphiprion bicinctus*) and bulb-tentacle sea anemones (*Entacmaea quadricolor*) at Auburn University. Plot depicts the oxygen concentrations of seawater passing electrode 1 (immediately before entering the respirometry chamber) and electrode 2 (immediately after exiting the chamber). Letters indicate the time at which experimental animals were added to the chamber (a), and the time at which standard metabolic rate was achieved (b). Oxygen uptake rate of the experimental animals was derived from the mean difference between the two electrode readings for 20 min after time b.

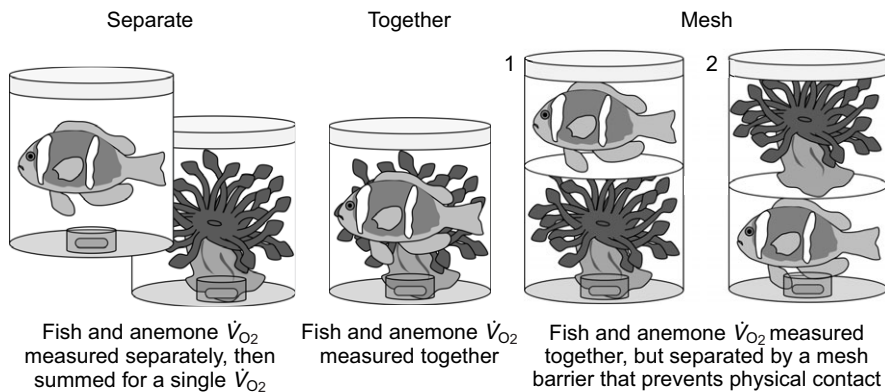


Fig. 2. Flow-through respirometry treatments to assess the effects of symbiotic interactions on the dark oxygen uptake ( $\dot{V}_{O_2}$ ) of two-band anemonefish (*A. bicinctus*) and bulb-tentacle sea anemones (*E. quadricolor*). Mesh subtreatments, mesh<sub>1</sub> (1) and mesh<sub>2</sub> (2), are depicted. Respirometry chambers were constructed from 6.35-mm-thick acrylic cylinders (inner diameter, 16.5 cm) with a volume of either 2.5 or 3.5 l (depending on animal sizes).

The dark  $\dot{V}_{O_2}$  of fish–anemone pairs was measured during three experimental treatments applied in random order (Fig. 2): (1) the ‘separate’ treatment measured the dark  $\dot{V}_{O_2}$  of the fish and anemone separately, and the two rates were added together for a single net dark  $\dot{V}_{O_2}$ ; (2) the ‘together’ treatment measured the net dark  $\dot{V}_{O_2}$  of the fish and anemone within the chamber as a united pair; and (3) the ‘mesh’ treatment measured the net dark  $\dot{V}_{O_2}$  of the fish and anemone as a pair within the same chamber, but physically separated by a flow-permeable screen (1 mm mesh), which permitted visual and chemical interaction while preventing any physical contact between partners. Because of time and collection limitations, the dark  $\dot{V}_{O_2}$  of wild animals at the Marine Science Station was measured during only the first two treatments (separate and together), and a single anemone was used with all fish examined ( $N=6$ ). The dark  $\dot{V}_{O_2}$  of cultured animals at Auburn University was measured across all three treatments, and each pair ( $N=12$ ) consisted of a unique fish and anemone. Furthermore, a subsample of cultured pairs ( $N=6$ ) was subjected to the mesh treatment twice; once with the anemone in the bottom of the chamber such that incoming seawater passed by the anemone before reaching the fish (mesh<sub>1</sub>), and again with the fish in the bottom of the chamber such that incoming seawater first passed by the fish before reaching the anemone (mesh<sub>2</sub>). The mean  $\dot{V}_{O_2}$  difference between mesh<sub>1</sub> and mesh<sub>2</sub> was then compared to assess whether (a) basal nitrogen or other dissolved substances released by fish (Roopin et al., 2011) affected anemone  $\dot{V}_{O_2}$  (mesh<sub>2</sub>>mesh<sub>1</sub>), or (b) dissolved substances released by anemones (Arvedlund et al., 1999) affected fish  $\dot{V}_{O_2}$  (mesh<sub>1</sub>>mesh<sub>2</sub>). Because of differences in design between the experiments conducted on wild animals at the Marine Science Station and cultured animals at Auburn University, direct comparisons between wild and cultured pairs were not made.

#### Effects of water motion on anemone nocturnal oxygen uptake

The effects of water flow on the dark  $\dot{V}_{O_2}$  of cultured anemones ( $N=8$ ) were also assessed using flow-through respirometry. Anemones were transferred to a respirometry chamber and acclimated for 3–6 h, as described above. Water flow regimes were created by varying the speed of the caged stir bar within the empty chambers in addition to secondary water flow created by the inflow and outflow ports on opposite sides of the chamber. We estimated the nominal velocity both in the center and near the edges of the chambers using a Flo-Mate 2000 portable flow meter (Marsh-McBirney, Frederick, MD, USA) and found no clear differences in velocity between these areas. Anemones were exposed to nine flow speeds (0.5–8.0 cm s<sup>-1</sup>) commonly encountered by benthic

organisms on coral reefs in the northern Red Sea (Goldshmid et al., 2004). At each flow speed, 10 min of  $\dot{V}_{O_2}$  was measured (10 measurements min<sup>-1</sup>, see above). The maximum O<sub>2</sub> uptake rate achieved and the maximum difference in O<sub>2</sub> uptake between the lowest and highest flow speeds were calculated.

#### Anemonefish nocturnal behavior

##### Fish behaviors in respirometry chambers

Possible effects of fish behavior on the net dark  $\dot{V}_{O_2}$  of the symbiotic pairs were investigated by observing the nocturnal activity of the fish during the 20 min of  $\dot{V}_{O_2}$  used for respirometry analysis for each respirometry treatment (separate, together, mesh<sub>1</sub>, mesh<sub>2</sub>) using a Sony DCR-SR68 IR video camera (Sony, San Diego, CA, USA) and IRLamp6 LED infrared lamps (Wildlife Engineering, Carlisle, PA, USA). From each 20 min segment, one random 5 min subsegment was observed per fish per treatment to (a) categorize the behavioral repertoire of fish at night, and (b) determine the effects of anemone presence on fish behavior. We assumed that these 5 min periods were representative of longer periods, based on previous behavioral observations of anemonefishes (Green and McCormick, 2004).

The percentage time and bout frequency of five distinct fish behaviors were measured: fanning, wedging, switching, swimming and no motion (see supplementary material Movie 1). Fanning was defined as the fish remaining completely motionless except for continuous pectoral fin strokes. Wedging was a brief (1–2 s) behavior in which fish used rapid fin strokes and lateral undulation of the posterior musculature to forcefully wiggle deeper among the anemone tentacles or toward the bottom of the aquarium, if the anemone was absent (Allen, 1975). Switching was a modified form of wedging, in which the fish changed body orientation (usually ~180 deg) while wiggling deeper among the anemone tentacles or toward the aquarium bottom (‘shifting’) (Allen, 1975). Swimming was defined as the fish rising up and entering the water column to forage or roam. Finally, no motion was defined as the fish resting completely motionless on the aquarium bottom or the anemone tentacle crown. Additional behavioral observations were conducted in the 75 l holding aquaria of each fish–anemone pair to ensure that the chamber setting did not alter fish behavior.

##### Fish nocturnal fin stroke frequencies

The effects of anemone presence on the fin stroke frequency (pectoral and caudal) of fish at night were also measured using five random 5 s video segments (25 s per fish per treatment) selected from the middle of the night (24:00 h–03:00 h). Six fish–anemone pairs were selected randomly from a pool of 13 cultured anemonefish



and 12 anemones. In random order, fish were observed in each of two treatments: anemone absent and present. For each treatment, the fish (or fish and anemone) was transferred in a glass beaker to an experimental aquarium (75l) and acclimated for 24h. Eight 20min video segments (1h apart) were recorded automatically throughout the night (20:00h–06:00h) per fish per treatment. We assumed that these 5s periods were representative of fin stroke frequencies over longer periods, based on previous methods (Goldshmid et al., 2004).

#### Statistical analysis

Statistical analyses were conducted using SAS 9.2 (Cary, NC, USA). Differences in fish and anemone dark  $\dot{V}_{O_2}$  among respirometry treatments, and the effects of water flow speed (semi-ln transformed) on anemone dark  $\dot{V}_{O_2}$ , were examined using one-way repeated-measures analysis of variance (rmANOVA). The dark  $\dot{V}_{O_2}$  of mesh<sub>1</sub> and mesh<sub>2</sub> sub-treatments was compared using a paired *t*-test. The effects of treatment (anemone absent and present) and the effects of anemone presence on fish fin stroke frequency (pectoral and caudal) were analyzed using one- or two-way rmANOVA. The effects of treatment on fish behavior within the respirometry chambers were analyzed with a non-parametric Friedman's Chi square test. Where appropriate, *post hoc* multiple pairwise comparisons were made using Tukey's studentized range (HSD) tests. For rmANOVA models, where the assumption of sphericity was not met, Greenhouse–Geisser approximations were used. The significance level for all analyses was set at  $P < 0.05$ . All reported values are means  $\pm$  1 s.e.m.

## RESULTS

### Patterns of nocturnal oxygen uptake

Symbiotic associations between anemonefish and sea anemones significantly affected their metabolism. The net dark  $\dot{V}_{O_2}$  of the symbiotic partners when together in the experimental chamber (together treatment) was 1.4 times higher on average than the summed  $\dot{V}_{O_2}$  of the isolated partners (separate treatment) for both wild (Table 1A) and cultured pairs (Table 1B). Moreover, physical contact between the partners was required to induce this increased  $\dot{V}_{O_2}$  when partners were together. Dark  $\dot{V}_{O_2}$  was significantly higher when physical contact between partners was allowed (together treatment) than when not allowed (mesh treatment) (Table 1B). Also, dark  $\dot{V}_{O_2}$  between the separate and mesh treatments did not differ significantly (Table 1B), and the position of each partner in the chamber during the mesh<sub>1</sub> and mesh<sub>2</sub> treatments had no significant effect on net dark  $\dot{V}_{O_2}$  of the partners (Table 1C).

### Effects of water motion on anemone nocturnal $\dot{V}_{O_2}$

Sea anemone  $\dot{V}_{O_2}$  increased significantly with increasing water flow rates from 0.5 to 2.0 cm s<sup>-1</sup> (85.93 $\pm$ 11.07 to 108.89 $\pm$ 12.59  $\mu$ mol O<sub>2</sub> h<sup>-1</sup>) but then remained constant from 2.0 to 8.0 cm s<sup>-1</sup> (rmANOVA,  $F=41.32$ ,  $P < 0.0001$ ) (Fig. 3). The maximum anemone  $\dot{V}_{O_2}$  reached was 114.21 $\pm$ 13.89  $\mu$ mol O<sub>2</sub> h<sup>-1</sup>, and the maximum difference in  $\dot{V}_{O_2}$  across flow regimes was 23.69 $\pm$ 2.64  $\mu$ mol O<sub>2</sub> h<sup>-1</sup>.

### Anemonefish nocturnal behavior

In respirometry chambers, all fish spent 80.11 $\pm$ 16.10% of the night in one location, regardless of the treatment. When an anemone was present, it always served as this location. In the absence of an anemone, fish rested on the floor of the chamber. Despite this limited locomotion, fish engaged in a myriad of localized behaviors throughout the night. Additional behavioral observations conducted in the 75l holding aquaria of each fish–anemone pair did not differ significantly from the behaviors observed in the chambers.

### Percentage time of anemonefish behaviors

Symbiotic association between fish and anemones influenced the percentage time that fish engaged in wedging and switching behaviors. Fish spent much more time wedging (20 times) and switching (2.5 times) when an anemone was accessible (together treatment) than when it was either absent (separate treatment) or inaccessible (mesh<sub>1</sub> and mesh<sub>2</sub> treatments; Table 2A). In contrast, access to an anemone had no effect on the percentage time that fish spent fanning, swimming or not moving in respirometry chambers (Table 2A).

### Frequencies of anemonefish behaviors

Access to an anemone significantly increased the frequencies of some behavioral changes by fish. When an anemone was physically accessible in the chamber (together treatment), fish significantly increased the frequency of fanning (3 times), wedging (6 times) and switching (30 times) (Table 2B). Fish engaged in bouts of switching more frequently during the mesh<sub>1</sub> treatment (fish downstream of anemone, 3.00 $\pm$ 1.26 bouts 5 min<sup>-1</sup>) than during the separate and mesh<sub>2</sub> treatments (0.00 bouts 5 min<sup>-1</sup>). In contrast, bout frequencies of swimming and no motion were unaffected by access to an anemone (Table 2B).

The pectoral fin stroke frequencies of fish at night did not vary with anemone presence (0.69 $\pm$ 0.09 Hz when anemone present *versus* 0.75 $\pm$ 0.04 Hz when absent, rmANOVA,  $F=0.32$ ,  $P=0.32$ ) (Fig. 4). Nocturnal caudal fin stroke frequencies were significantly more rapid when fish were with host anemones (0.26 $\pm$ 0.06 Hz) than without (0 Hz) (rmANOVA,  $F=14.29$ ,  $P=0.013$ ).

Table 1. Repeated-measures ANOVA on effects of respirometry treatment on dark oxygen uptake ( $\dot{V}_{O_2}$ )

	Treatment	$\dot{V}_{O_2}$ ( $\mu$ mol O <sub>2</sub> h <sup>-1</sup> )	d.f.	MS	<i>F</i>	<i>P</i>
A	Separate	163.62 $\pm$ 4.78	1	10,168.578	16.26	<b>0.01</b>
	Together	221.27 $\pm$ 12.24				
B	Separate	213.84 $\pm$ 14.92	2	24,266.53	19.22	<b>&lt;0.001</b>
	Together	283.10 $\pm$ 14.08				
	Mesh <sub>1</sub> *	203.14 $\pm$ 10.71				
C	Mesh <sub>1</sub>	209.78 $\pm$ 13.99	1	351.84	0.29	0.6
	Mesh <sub>2</sub>	198.95 $\pm$ 14.55				

Experiments were carried out on two-band anemonefish (*Amphiprion bicinctus*) and bulb-tentacle sea anemones (*Entacmaea quadricolor*) at the Marine Science Station in Aqaba, Jordan (A, wild) and at Auburn University in Alabama, USA (B,C, cultured).

Data are means  $\pm$  1 s.e.m. Significant results are in bold.

\*No difference in  $\dot{V}_{O_2}$  between mesh treatments (C), so only mesh<sub>1</sub> is presented.

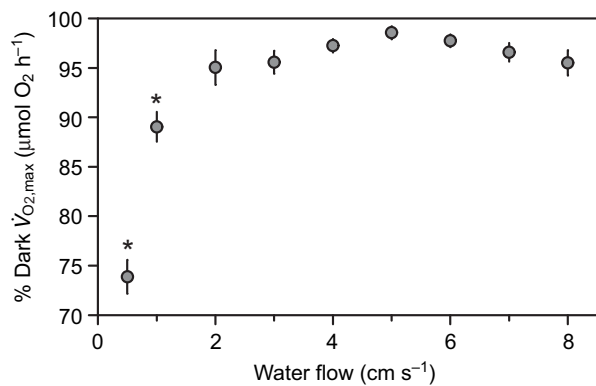


Fig. 3. Effects of water flow rate on the dark maximum oxygen uptake ( $\dot{V}_{O_2,max}$ ; mean  $\pm$  1 s.e.m.) of bulb-tentacle sea anemones (*E. quadricolor*) in flow-through respirometry. Mean  $\dot{V}_{O_2,max}$  = 114.21  $\pm$  13.89  $\mu\text{mol O}_2 \text{ h}^{-1}$ .  $\dot{V}_{O_2,max}$  values at 0.5 and 1.0  $\text{cm s}^{-1}$  were significantly different (\* $P < 0.05$ ).

## DISCUSSION

### Effects of symbiotic association on oxygen uptake

We demonstrate here that the symbiotic association between two-band anemonefish and bulb-tentacle sea anemones enhances the dark  $\dot{V}_{O_2}$  of one or both partners at night, and that physical contact between the partners stimulates this enhanced dark  $\dot{V}_{O_2}$ . While we did not discern the relative importance of each partner's contribution to this metabolic elevation, it is likely that anemonefish behavior improves sea anemone  $\dot{V}_{O_2}$ , because (a) sea anemone  $\dot{V}_{O_2}$  increases with water flow, and (b) anemonefish engage in more flow-modulating behaviors when they associate with sea anemones than when alone.

The changes observed here in the nocturnal behaviors of anemonefish that are induced by the presence of sea anemone hosts appear to enhance water motion among sea anemone tentacles. Firstly, we observed that sea anemone tentacles appear to move more when anemonefish are present than when absent, although we did not quantify this behavior. Secondly, rapid and forceful bouts of wedging and switching by anemonefish likely increase the shear and turbulence of ambient water, and augment gas exchange across sea anemone tissues. However, enhanced gas exchange by sea anemones likely is not wholly responsible for the  $\dot{V}_{O_2}$  elevation observed when the partners are together. The mean difference in  $\dot{V}_{O_2}$  of sea anemones between low and high water flow is less than

half the mean increase in net  $\dot{V}_{O_2}$  induced by physical contact between the symbiotic partners (29.41  $\pm$  3.58 versus 70.96  $\pm$  10.03  $\mu\text{mol O}_2 \text{ h}^{-1}$ , respectively). Thus, the metabolic elevation observed here during the together treatment is too large to be achieved by sea anemones alone, and elevated anemonefish metabolism (e.g. increased activity) is also required to explain the total increase in  $\dot{V}_{O_2}$  when the partners are together.

Our quantitative laboratory observations, in contrast to qualitative field observations on other anemonefish species (Allen, 1975), indicate that anemonefish spend most of the night in some form of motion. Possible reasons for this discrepancy may be that some anemonefish behaviors are subtle and difficult to quantify in the field, or that some anemonefish species move more at night than do others (Allen, 1975). The total percentage time spent in motion by anemonefish at night does not appear to depend on sea anemone presence, and their fin stroke frequencies are substantially lower than those of other damselfish species that reside among coral hosts at night (Goldshmid et al., 2004). However, our data indicate that anemonefish alternate their behaviors substantially more frequently when sea anemones are present than when not, and also increase both the percentage time and bout frequencies of certain behaviors when they interact with the host (i.e. wedging and switching).

We propose that wedging and switching by anemonefish may function in part to aerate host anemones, because both of these behaviors involve rapid caudal and pectoral fin movement, and act to forcefully propel anemonefish deep within the sea anemone tentacle crown. These vigorous behaviors rely heavily upon the caudal fin and posterior musculature, and likely require more energy than do the other fish behaviors observed here. In contrast, bouts of fanning involve no fish movement, aside from alternating pectoral fin strokes, and swimming typically involves only simultaneous pectoral fin rowing. Other types of fishes that associate with reef cnidarians also exhibit high levels of nocturnal activity that appear to aerate their hosts (Holbrook and Schmitt, 1997; Goldshmid et al., 2004).

Thus, increased instances of wedging and switching by anemonefish when united with host anemones potentially (a) elevate the energy expenditure of anemonefish through increased activity, and (b) increase sea anemone gas exchange through enhanced ambient water flow. Together, these effects provide a likely explanation for the increased net dark  $\dot{V}_{O_2}$  of the anemonefish and sea anemone partners during the together treatment.

Table 2. Anemonefish behaviors

Behavior	Treatment				Friedman's Chi square test			
	Separate	Together	Mesh <sub>1</sub>	Mesh <sub>2</sub>	d.f.	Q	P	
A	Fanning	59.56 $\pm$ 17.55	79.00 $\pm$ 9.10	79.06 $\pm$ 15.87	70.33 $\pm$ 15.40	3	2.39	0.496
	Wedging	0.33 $\pm$ 0.33	8.39 $\pm$ 1.57	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	3	18.85	<b>&lt;0.001*</b>
	Switching	0.00 $\pm$ 0.00	2.61 $\pm$ 0.71	1.06 $\pm$ 0.44	0.00 $\pm$ 0.00	3	14.36	<b>0.003*</b>
	Swimming	5.78 $\pm$ 3.45	0.00 $\pm$ 0.00	19.89 $\pm$ 16.10	17.44 $\pm$ 16.53	3	5.03	0.170
	No motion	34.33 $\pm$ 15.44	10.00 $\pm$ 8.63	0.00 $\pm$ 0.00	12.22 $\pm$ 7.01	3	4.36	0.225
B	Fanning	4.67 $\pm$ 1.65	17.67 $\pm$ 3.85	4.50 $\pm$ 1.31	3.33 $\pm$ 1.15	3	10.16	<b>0.017*</b>
	Wedging	0.50 $\pm$ 0.50	15.17 $\pm$ 6.86	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	3	12.75	<b>0.005*</b>
	Switching	0.00 $\pm$ 0.00	6.00 $\pm$ 1.39	3.00 $\pm$ 1.27	0.00 $\pm$ 0.00	3	14.36	<b>0.003**</b>
	Swimming	1.00 $\pm$ 0.45	0.00 $\pm$ 0.00	1.00 $\pm$ 0.37	0.33 $\pm$ 0.21	3	7.69	0.053
	No motion	5.00 $\pm$ 2.42	3.67 $\pm$ 2.89	0.00 $\pm$ 0.00	2.50 $\pm$ 1.15	3	4.36	0.225

Friedman's Chi square test on effects of respirometry treatments (Fig. 2) on the percentage time (A) and bout frequency (bouts 5 min<sup>-1</sup>, B) of five types of nocturnal behavior of two-band anemonefish (*Amphiprion bicinctus*).

Significant results are in bold.

\*Together treatment was significantly higher than separate, mesh<sub>1</sub> and mesh<sub>2</sub> treatments.

\*\*Together treatment was significantly higher than mesh<sub>1</sub>, which was significantly higher than separate and mesh<sub>2</sub> treatments.

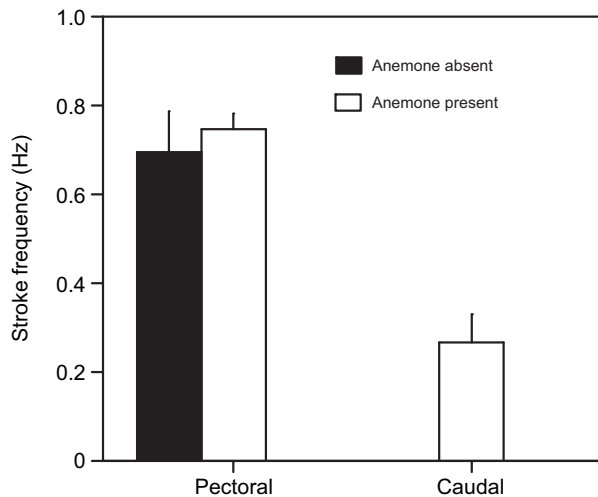


Fig. 4. Nocturnal stroke frequencies (means and 1 s.e.m.) of the dorsal and caudal fins of two-band anemonefish (*A. bicinctus*). Fin stroke frequencies were measured when anemonefish were alone or with bulb-tentacle sea anemones (*E. quadricolor*). No caudal fin strokes occurred in the absence of an anemone (significantly different from caudal fin strokes with anemone present).

#### Implications for the mutualism

Water flow is one of the most important abiotic factors affecting the growth and survivorship of sessile marine invertebrates (Sebens et al., 2003), which generally lack the ability to efficiently self-regulate the mass transfer of dissolved particles across their tissues (Shick, 1990). The flow-induced reduction or elimination of the diffusive boundary layer surrounding sedentary organisms notably enhances gas exchange (Patterson and Sebens, 1989; Patterson et al., 1991; Bruno and Edmunds, 1998; Sebens et al., 2003; Finelli et al., 2006; Schutter et al., 2010), nutrient uptake (Stambler et al., 1991; Atkinson and Bilger, 1992; Lesser et al., 1994; Thomas and Atkinson, 1997), prey capture (Helmuth and Sebens, 1993; Sebens, 1997; Sebens et al., 1998) and debris removal (Nugues and Roberts, 2003; Box and Mumby, 2007). Thus, anemonefish-enhanced water flow among sea anemone tentacles could provide several types of distinct benefits to the physiology and biology of the cnidarian host. In the present study, sea anemone dark  $\dot{V}_{O_2}$  increased with water flow, suggesting that respiration by these anemones is affected by flow rates. However, further research is needed to more accurately quantify the affect of flow regime and type on anemone gas exchange. Regardless, the finding here is supported by previous research showing that water flow decreases boundary layer thickness and increases gas exchange in the temperate sea anemone *Metridium senile* (Patterson and Sebens, 1989).

Physical contact between anemonefishes and sea anemones appears to be required for enhanced sea anemone gas exchange; however, chemical compounds released by sea anemones may play a role in initiating the anemonefish behaviors responsible for elevation of net  $\dot{V}_{O_2}$  when partners are together. For example, anemonefish engage in switching behaviors significantly more frequently when positioned downstream of sea anemones (mesh<sub>1</sub> treatment) than when positioned upstream (mesh<sub>2</sub> treatment). Dissolved chemical compounds released by anemones directly influence the recruitment to and recognition of sea anemones by anemonefishes (Murata et al., 1986; Arvedlund et al., 1999); however, the extent to which sea anemone chemical cues influence anemonefish behavior at night has yet to be determined.

While this study suggests that anemonefish behaviors modulate the hydrodynamic conditions surrounding sea anemones, it is unclear to what extent flow modulation is the major function of these behaviors. Sea anemones expand in the presence of anemonefishes, and contract when the anemonefishes are experimentally removed (Porat and Chadwick-Furman, 2004). Further, sea anemone body size is positively correlated with the size and quantity of anemonefish residents (Holbrook and Schmitt, 2005). Thus, anemonefish wedging and switching behaviors may influence the size and/or morphology of their hosts (Fautin Dunn, 1981). If anemonefish behavior promotes sea anemone expansion, this process may increase sea anemone  $\dot{V}_{O_2}$  by exposing more tissue surface area for gas exchange. Alternatively, some coral reef fishes receive health benefits from simple tactile stimulation by their symbiotic partners (Soares et al., 2011). Anemonefishes kept in captivity without sea anemone hosts occasionally bathe in airstream bubbles and stringy algal tufts, and may engage in these behaviors because tactile stimulation from anemone tentacles benefits the 'well-being' of the fishes (Mariscal, 1970b). More research is needed to clarify the factors that enhance the expression of certain behaviors (i.e. wedging, switching) when anemonefishes reside among sea anemone tentacles. Wild individuals of both *A. bicinctus* and *E. quadricolor* attain much larger maximum sizes than the individuals examined here, and in the Red Sea, each individual of *E. quadricolor* usually hosts a pair of adult fish plus up to three juveniles (Chadwick and Arvedlund, 2005; Huebner et al., 2012). The ecophysiological effects of anemonefish size, quantity and social structure on anemone hosts in the wild are largely unknown, and further investigation is needed to clarify the nocturnal behaviors of anemonefish in the wild.

#### CONCLUSIONS

Symbiotic partners on coral reefs provide a myriad of ecophysiological benefits that buffer their associates against environmental stressors, such as  $O_2$  variability and water flow patterns. Our findings demonstrate that the association between anemonefish and sea anemones elevates the  $\dot{V}_{O_2}$  of the symbionts at night, regardless of animal origin (i.e. wild or captive). We also show that the presence of host sea anemones alters anemonefish behavior, and that certain fish behaviors (i.e. wedging and switching) appear to modulate water flow among sea anemone tentacles, and potentially increase both anemonefish and sea anemone oxygen uptake. These results provide foundational evidence of anemonefish-induced flow modulation around sea anemone hosts, a previously debated benefit of this mutualism. Further, this study documents metabolic consequences to both partners of anemonefish behavior at night, and thus joins a growing body of evidence indicating the importance of physiological mechanisms in the ecological benefits provided by coral reef mutualisms. Further investigation of the ecophysiology and behaviors of mutualists on coral reefs will clarify how reef organisms adapt to changes in their naturally variable environment.

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