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Hormonal and behavioural effects of motorboat noise on wild coral reef fish[☆]

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ABSTRACT

Anthropogenic noise is an emergent ecological pollutant in both terrestrial and aquatic habitats. Human population growth, urbanisation, resource extraction, transport and motorised recreation lead to elevated noise that affects animal behaviour and physiology, impacting individual fitness. Currently, we have a poor mechanistic understanding of the effects of anthropogenic noise, but a likely candidate is the neuroendocrine system that integrates information about environmental stressors to produce regulatory hormones; glucocorticoids (GCs) and androgens enable rapid individual phenotypic adjustments that can increase survival. Here, we carried out two field-based experiments to investigate the effects of short-term (30 min) and longer-term (48 h) motorboat-noise playback on the behaviour, GCs (cortisol) and androgens of site-attached free-living orange-fin anemonefish (*Amphiprion chrysopterus*). In the short-term, anemonefish exposed to motorboat-noise playback showed both behavioural and hormonal responses: hiding and aggression increased, and distance moved out of the anemone decreased in both sexes; there were no effects on cortisol levels, but male androgen levels (11-ketotestosterone and testosterone) increased. Some behaviours showed carry-over effects from motorboat noise after it had ceased, and there was no evidence for a short-term change in response to subsequent motorboat-noise playback. Similarly, there was no evidence that longer-term exposure led to changes in response: motorboat noise had an equivalent effect on anemonefish behaviour and hormones after 48 h as on first exposure. Longer-term noise exposure led to higher levels of cortisol in both sexes and higher testosterone levels in males, and stress-responses to an additional environmental challenge in both sexes were impaired. Circulating androgen levels correlated with aggression, while cortisol levels correlated with hiding, demonstrating in a wild population that androgen/glucocorticoid pathways are plausible proximate mechanisms driving behavioural responses to anthropogenic noise. Combining functional and mechanistic studies are crucial for a full understanding of this global pollutant.

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1. Introduction

Anthropogenic noise is a well-recognised global pollutant that has a wide range of effects on wildlife, but the proximate

mechanisms for its impact are considered relatively rarely. Human activities that generate sound, either deliberately or incidentally, have increased since the Industrial Revolution as a consequence of, for instance, the growth in urbanisation, resource extraction, transport networks and motorised recreation (Hildebrand, 2009; Buxton et al., 2017). Both terrestrial and aquatic soundscapes have been altered worldwide, with those man-made sounds which contain little or no useful information, and which have negative effects on wildlife, viewed as noise pollution. The last 15 years has seen a rapid increase in studies demonstrating impacts of

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anthropogenic noise on a diverse array of taxa: including, for example, shifts in hearing thresholds, physical and physiological damage, masking of acoustic cues and signals, and alterations both to individual behaviour and to conspecific and heterospecific interactions (Morley et al., 2014; Shannon et al., 2015; Hawkins & Popper, 2017). These can translate into consequences for individual fitness, and potentially populations and communities (Francis et al., 2011; Simpson et al., 2016; Day et al., 2017; Nedelec et al., 2017a). However, the proximate mechanisms for such noise effects are less clear and are rarely explored experimentally in wild systems.

The neuroendocrine control system provides a flexible link between changes in the environment and appropriate morphological, physiological and behavioural responses (Adkins-Regan, 2005; Bonier, 2012). One of the pivotal components of the neuroendocrine system, the hypothalamic–pituitary–adrenal (HPA) axis, involves three responses: a primary response (initial neuroendocrine response) followed by secondary responses (physiological adjustments) and finally tertiary responses (whole-animal performance) (Barton, 2002; Portz et al., 2006). Once triggered by environmental challenges, the HPA axis releases glucocorticoid hormones (GCs) (primary response) that have multiple regulatory effects on an individual's biology (secondary and tertiary responses), including suppression of the hypothalamic–pituitary–gonadal (HPG) axis that regulates the release of androgens such as testosterone (Sapolsky et al., 2000; Wingfield & Sapolsky, 2003; Boonstra, 2004). GCs therefore play a crucial role in enabling vertebrates to cope with, and respond to, environmental stressors in the wild (e.g. Romero & Wikelski, 2001; Kitaysky et al., 2007; Beldade et al., 2017). However, despite there being evidence that anthropogenic noise activates the stress axis (e.g. Kleist et al., 2018), direct links have rarely been made with subsequent secondary and tertiary responses.

Anthropogenic noise has been shown to stimulate the initial (baseline) hormonal response with elevations in circulating levels of GCs (birds: Hayward et al., 2011; Blickley et al., 2012b; mammals: Creel et al., 2002; amphibians: Tennesen et al., 2014; Kaiser et al., 2015; fishes: Wysocki et al., 2006; Nichols et al., 2015), but not in all cases (Crino et al., 2013; Angelier et al., 2016). Secondary physiological responses to anthropogenic noise have been recorded that may have been triggered by the primary stress response; for example, increases in glucose and haematocrit (Filiciotto et al., 2013), the heterophil-to-lymphocyte ratio (Campo et al., 2005), cardiac output (Graham & Cooke, 2008), and ventilation and metabolic rates (Purser et al., 2016; Radford et al., 2016; Simpson et al., 2016), as well as compromised hemolymph physiology, reduced hemocyte density and disruption to osmoregulation (Day et al., 2017). Tertiary stress responses to anthropogenic noise that may result from effects on the neuroendocrine system include changes in foraging (Purser & Radford, 2011; Wale et al., 2013), schooling (Hawkins et al., 2014), movement (Picciulin et al., 2010; Buscaino et al., 2010; Holmes et al., 2017), anti-predator behaviour (Simpson et al., 2015; Purser et al., 2016), courtship (Blickley et al., 2012a), parental care (Picciulin et al., 2010; Naguib et al., 2013), inter-species interactions (Nedelec et al., 2017a) and survival (Simpson et al., 2016; Day et al., 2017; Nedelec et al., 2017b). However, none of these studies have demonstrated the proximate links from primary to tertiary stress responses, thus rendering proximate mechanisms in response to anthropogenic noise difficult to elucidate.

Most experimental noise studies have considered immediate responses to single, relatively short-term exposures, but repeated exposure to noise could lead to a heightened or lessened response. Animal responses to noise could change with repeated stimulus exposure due to sensitisation, increased tolerance, habituation or

hearing-threshold shifts (Romero, 2004; Bejder et al., 2009; Radford et al., 2016). The few studies that have compared exposures to anthropogenic noise (acute, brief and transient versus chronic, longer, cumulative and repetitive) have produced conflicting results, with a reduced response (Radford et al., 2016; Nedelec et al., 2016; Holmes et al., 2017), no difference in response (Bruintjes & Radford, 2013; Nedelec et al., 2017b; Schramm et al., 2017) or an increased response (Day et al., 2017) found in different cases. Moreover, such longer-term studies have not generally considered changes in the response of the HPA and HPG axes (GC and androgen responses). Therefore, experimental studies under natural conditions, aimed at determining the proximate mechanisms of anthropogenic noise and testing for changes in responses with longer-term exposure, are needed.

Here we examine the neuroendocrine (primary) and behavioural (tertiary) impacts of motorboat noise on wild pairs of site-attached orange-fin anemonefish, *Amphiprion chrysopterus* (Cuvier 1830), in the lagoonal reef system of Moorea, French Polynesia. Many tropical marine fish species are sessile or site-attached, in particular Pomacentridae such as anemonefish (e.g. *Amphiprion* sp.) and damselfish (e.g. *Dascyllus* sp.) that live in tight association with anemones or corals respectively; unable to move away from localised stressors, they are especially vulnerable to environmental perturbations. *Amphiprion chrysopterus* lives mostly in association with the anemone *Heteractis magnifica*, and is found across reef habitats (Beldade et al., 2016). *Amphiprion* sp. produce sound at frequencies from 370 to 900 Hz, and detect sounds between 75 and 1800 Hz (Parmentier et al., 2009); a previous study found the dominant frequency of the vocalisations of an *A. chrysopterus* to be 411 Hz (Colleye et al., 2011), overlapping with the broadband sound produced by motorboats. Their close association with anemones renders *A. chrysopterus* a tractable species for field studies of behavioural and hormonal responses (Mills et al., 2010; Mills et al., 2015; Beldade et al., 2017; Mills et al., 2018).

We carried out two field-based experiments to investigate the effects of short-term (30 min) and longer-term (48 h) motorboat-noise playback on male and female anemonefish behaviour, GCs and androgens. First, we consider how natural behaviours around the host anemone (hiding, distance from anemone, aggression towards heterospecifics) are affected by initial exposure to motorboat-noise playback, the possibility of carry-over effects once the noise stimulus stops, and potential changes in responses with repeated exposure. Second, we examine the hormonal responses to motorboat-noise playback over both timeframes, to determine the GC and androgen pathways involved in regulating the observed behavioural effects. We predicted that short-term exposure to motorboat-noise playback would cause a change in behaviour (e.g. increased hiding and aggression, decreased distance moved), GCs (increased cortisol) and androgens (increased 11-KT and T), but such changes would no longer be manifest after longer-term exposure. Moreover, we predicted that cortisol levels would be related to both hiding and distance from anemone as stressed fish often exhibit riskier behaviour relative to unstressed conspecifics (Brown et al., 1985; Piatto et al., 2011). In addition to baseline cortisol, we predicted that exposure to motorboat noise would impact stress-induced cortisol indicating that the HPA (or hypothalamic–pituitary–interrenal (HPI) axis for fish) has been compromised. We also predicted that 11-ketotestosterone and testosterone would be related to aggression levels as both are important in regulating territory defence (Desjardins et al., 2008; Mills et al., 2018).

2. Materials and methods

2.1. Experimental overview

Two field-based experiments were carried out on free-living pairs of orange-fin anemonefish in the northern lagoon of Moorea, French Polynesia (17°31'04.4"S 149°51'02.0"W; Fig. S1). Both experiments entailed playback of either ambient reef sound (control) or ambient reef sound and motorboat noise, in an independent-samples design.

Experiment 1 considered short-term effects of motorboat noise; experimental trials lasted 1 h (Fig. S2). An underwater loudspeaker was placed 1 m from the focal anemone to which a pair of anemonefish was associated (Fig. S3); pairs were assigned alternately to ambient and motorboat treatments when possible (equipment failure notwithstanding) ($N = 20$ in each case). Focal anemones were in seven zones around Moorea with, on average, equal numbers of both treatments in each zone (Table S1a; Fig. S1). Trials were carried out over 76 days between March 30, 2014 and June 11, 2014 from 09h10–13h30 (Table S1a), with no significant difference between treatments in the date (Mann Whitney U tests: $U = 194.0$, $N = 40$, $p = 0.871$) or the time of day ($U = 176.5$, $N = 40$, $p = 0.524$). The loudspeaker was switched on to play a silent track (a 30 min period of silence on all playback tracks) and, after a 10 min acclimation period to the presence of the loudspeaker and a snorkeler 3 m from the host anemone (Fig. S3) (Nanninga et al., 2017), the baseline behaviour of both the male and female fish (sex determined by size; Fricke, 1979; Buston, 2003) was recorded for 20 min (see 'Behavioural data' below). A trial then continued with a 30 min sound-treatment track (see 'Playback tracks' below) consisting of either alternating 5 min periods of ambient-reef sound and ambient-reef sound plus motorboat noise (motorboat treatment) or alternating 5 min periods of different ambient-reef sound (ambient treatment). Behavioural observations of the focal pair were taken during each 5 min period. Twenty focal pairs ($N = 10$ per treatment; those that did not continue to receive playbacks in Experiment 2) were then targeted for hormonal blood sampling (final sample sizes vary depending on capture success and the time taken to capture and bleed fish; see 'Hormonal data' below). All fish sampled for hormones were returned to their host anemone immediately afterwards and recovered normal behaviours.

Experiment 2 considered longer-term effects of motorboat noise; experimental trials lasted 2 days (Fig. S2). Twenty anemonefish pairs from Experiment 1 ($N = 10$ per treatment) continued to receive playbacks, from the underwater loudspeaker placed 1 m from the host anemone, over a 48 h period; sound treatment to each pair was as for Experiment 1. Focal anemones were in three zones around Moorea with, on average, equal numbers of both treatments in each zone (Table S1b; Fig. S1). Trials were carried out over 49 days between March 30, 2014 to May 15, 2014 (Table S1b), with no significant difference between treatments in the date (Mann Whitney U test: $U = 39.5$, $N = 20$, $p = 0.425$). Pairs were exposed continuously during daylight hours to relevant 30 min sound-treatment tracks on repeat. As motorboats are active at the study site only during the daylight period, sound-treatment tracks were timed to play between 06:00 and 18:00 for ecological validity. Over the 48 h trial period, motorboat-treatment pairs therefore received approximately 24 h of playback noise; loudspeakers were silent at night. At the end of 48 h, and after a 10 min acclimation period to the presence of a snorkeler conducting behavioural observations (Nanninga et al., 2017), the behaviour of the focal pair was recorded during a final 30 min playback period, as per Experiment 1. Behavioural observations were made from 09h10–13h30 (Table S1b), with no significant difference between treatments in

the time of day ($U = 47.0$, $N = 20$, $p = 0.819$). When possible, the focal fish (20 pairs) were then caught and bled for hormonal sampling (final sample sizes vary; see 'Hormonal data' below). All fish sampled for hormones were returned to their host anemone immediately afterwards and recovered normal behaviours.

2.2. Playback tracks

Playback tracks were constructed from original sound recordings made at the study site (Nedelec et al., 2014; Nedelec et al., 2016; SI 1). Daytime motorboat recordings were made of 36 passes by two motorboats with 25 hp Yamaha outboard engines 10–50 m from a hydrophone (HiTech HTI-96-MIN with inbuilt preamplifier; sensitivity –165 dB re 1V/ μ Pa; frequency range 2 Hz–30 kHz; High Tech Inc., Gulfport MS) and a solid-state recorder (Edirol R-09HR 16-bit recorder; sampling rate 44.1 kHz; Roland Systems Group, Bellingham WA). Twelve ambient-sound recordings (without motorboats) were also taken at the same location on each day. Each motorboat-pass recording was a sample lasting 40–50 s; ambient-sound recordings were 60 s. Thirty-minute ambient playback tracks contained only local ambient reef sound (6×5 min periods); 30 min motorboat tracks contained 3×5 min ambient-sound periods and 3×5 min motorboat-noise periods, the latter each with 6–7 motorboat passes. Two replicate 30 min playback tracks for each sound treatment were constructed from different recordings. All playback tracks included a 30 min period of silence prior to the 6×5 min periods of sound as both an acclimation period to the presence of the loudspeaker (Nanninga et al., 2017) and to collect baseline behaviour prior to any experimental sound. Fish experienced 18–21 and 425–511 motorboat passes in Experiment 1 and 2 respectively.

Playbacks to anemonefish were from underwater loudspeakers (UW-30, frequency response 0.1–10 kHz, University Sound, Columbus, USA) connected to MP3 players (Philips, Koninklijke, Netherlands) (Fig. S3). To compare playbacks with real sound sources, recordings of sound pressure were made using the hydrophone and solid-state recorder mentioned above, and recordings of particle acceleration were made using an accelerometer (M20-L Geospectrum Technologies, Dartmouth, Canada, sensitivity following a curve from 10 to 3000 Hz, calibrated by manufacturers) to a laptop via a USB soundcard (MAYA44, ESI Audiotechnik GmbH, Leonberg, Germany) (SI 1; Fig. 1). The root-mean-square sound-pressure levels (RMS SPL) for the frequency band 10–2000 Hz in dB re 1 μ Pa were as follows: ambient sound at site = 119; ambient playback = 121; motorboat playback (five passes) = 131; and real motorboat (five passes) = 131. The RMS particle-acceleration levels (PAL) for the two horizontal axes, for the same frequency band in dB re 1 μ m/s² were: ambient sound at site = 72; ambient playback = 70; motorboat playback (five passes) = 82; and real motorboat (five passes) = 89.

2.3. Behavioural data

Three behaviours were recorded during each relevant 5 min period (see 'Experimental overview' above), for the focal pair. (1) Percentage time spent hiding within the anemone; hiding defined as at least 50% of the body within the tentacles of the anemone. (2) Mean distance (m) from the focal anemone when outside the anemone. Distance from the focal anemone was estimated as the number of body lengths of the focal fish. After capture and measurement of individual fish (callipers: 0.1 cm), body-length estimates were converted to cm, and distance from anemone in m. The mean distance was determined from the time spent at each distance divided by the total time. (3) Number of aggressive acts directed towards heterospecifics (notably, juvenile three-spot

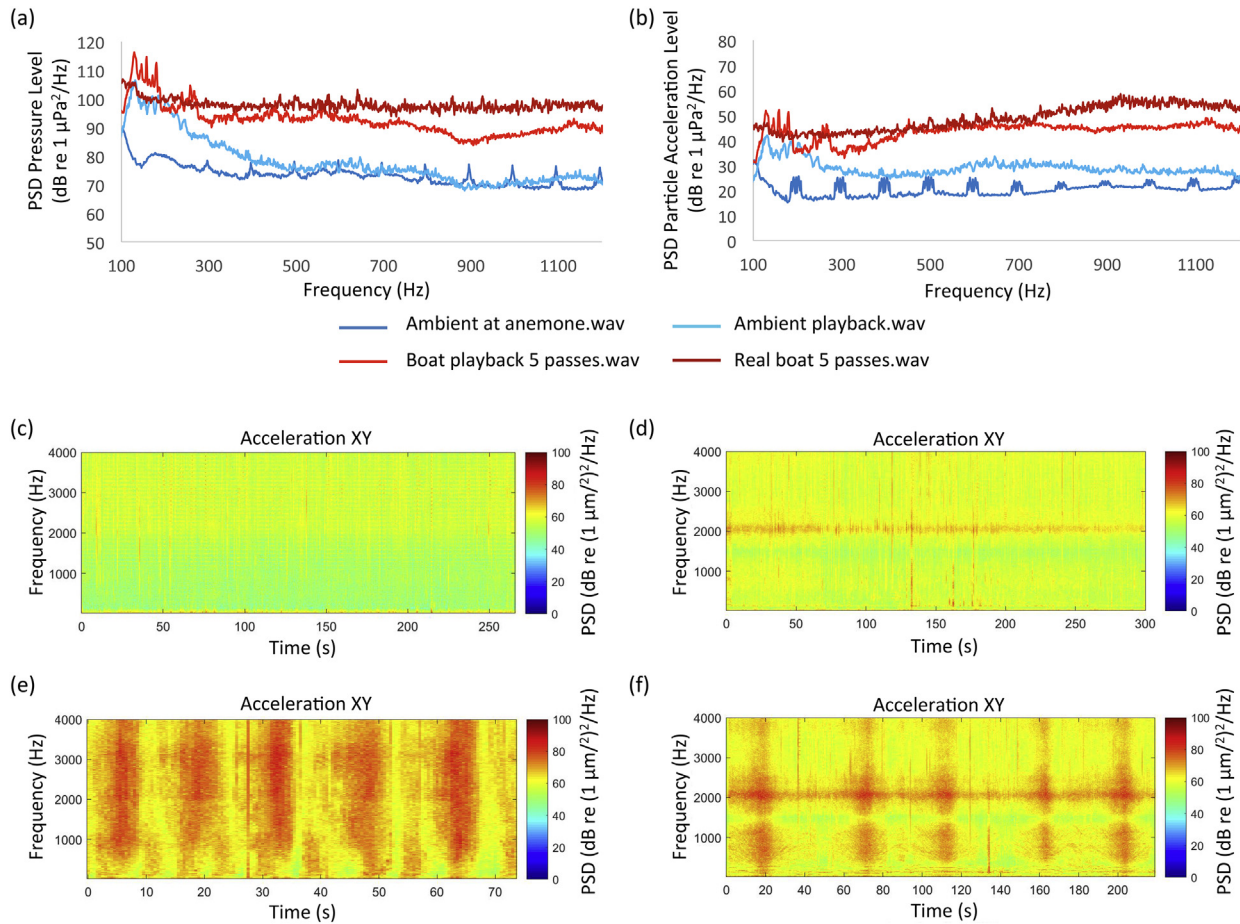


Fig. 1. Power spectral densities (PSDs) of a) sound-pressure level and b) dual-axis (two horizontal axes) particle acceleration for motorboat-noise and ambient-sound playbacks with a real motorboat and ambient sound at the study site for comparison. Recordings of playbacks were taken within 50 cm of anemonefish that were used in the experiment and at the same distance from the loudspeaker as the anemonefish. Recordings of the real motorboat were taken with the motorboat repeatedly passing the recording apparatus (distance = 10–100 m) in a boat channel. FFT length = 44,100, average of 5 min recordings for ambient-sound playback and 5 passes for motorboat-noise playback. Spectrograms of dual-axis particle acceleration for c) ambient sound with no playback at the study site, d) ambient-sound playback, e) real motorboat noise and f) motorboat-noise playback.

dascyllus, *Dascyllus trimaculatus*, which also use anemones as a nursery habitat but are an inferior competitor; O'Donnell et al., 2017). All behavioural observations in both experiments were made by a surface snorkeler 3 m from the focal anemone (SI 2).

The change or difference in each behaviour between particular 5 min observation periods, or simply the behaviour in a given period, was compared between the two sound treatments to test for the following effects.

- (i) The *baseline* behaviour of fish randomly allocated to the two sound treatments was assessed by considering the change from the final 5 min of silent playback to the first ambient-sound playback in Experiment 1 (B4 to 1st periods; Fig. S2).
- (ii) The *initial effect* of motorboat-noise playback was assessed by considering the change in Experiment 1 from the first ambient-sound period to the next 5 min period, which was either another ambient-sound period or a motorboat-noise period depending on sound treatment (1st to 2nd periods; Fig. S2).
- (iii) The potential for a *carry-over* effect of motorboat-noise playback immediately after it ceased was assessed by considering sound-treatment differences between the first and second ambient-sound periods in Experiment 1 (1st and 3rd periods; Fig. S2).

- (iv) The potential for a *short-term change in initial effect* to motorboat-noise playback with repeated intermittent exposure was assessed by considering the differences in Experiment 1 between the first and third motorboat-noise playback periods and their equivalents in the ambient-sound treatment (2nd and 6th periods; Fig. S2).
- (v) The *longer-term effect* of intermittent motorboat-noise playback on baseline behaviour was assessed by considering a sound-treatment difference in the initial ambient-sound period after 48 h of Experiment 2 (mth period; Fig. S2).
- (vi) The *effect of longer-term intermittent motorboat-noise exposure on the response to motorboat-noise playback* was assessed by considering the differences in Experiment 2 between the first ambient-sound period and the final 5 min period, which was either another ambient-sound period or the third motorboat-noise period depending on sound treatment (mth to nth periods; Fig. S2).

The behaviour of the male and female in a pair was often correlated (Table S2), so the sexes were analysed separately. Individuals in pairs were excluded from analysis if they laid eggs during the experiment (N = 3 pairs), electronic problems resulted in playbacks failing (N = 3 pairs) and if individuals were out of sight of snorkeller (N = 5 pairs). Therefore, in the short-term (30 min) exposure experiment (Experiment 1), the final sample sizes for

behavioural analyses were $N = 15$ pairs for the ambient-sound treatment and $N = 14$ pairs for the motorboat-noise treatment. In Experiment 2, the final sample sizes for behavioural analyses after 48 h were $N = 9$ pairs for the ambient-sound treatment and $N = 7$ pairs for the motorboat-noise treatment.

2.4. Hormonal data

Fish were captured underwater by scuba divers (SI 3). A blood sample of approximately 0.1 ml per fish (for baseline measurement of cortisol and the androgens: testosterone (T) and 11-ketotestosterone (11-KT)) was immediately collected laterally from the caudal vein using heparinised 1 ml syringes and kept on ice until processing. The time lapsed from first approaching the anemonefish until blood was flowing in the syringe was kept as short as possible (mean \pm SE = 4:51 min \pm 27 s; $N = 57$; SI 3).

In addition to baseline cortisol, the only effective way to assess whether an environmental stressor has compromised the stress axis is to measure additional indices (Norris & Evans, 2000) such as how the HPA (or HPI) axis responds to a standardised stressor. We measured maximal cortisol levels in response to a standardised stressor (stress-induced) which indicate how individuals would cope physiologically with environmental challenges re-directing energy and behaviours towards immediate survival (Wingfield et al., 1998). Each fish was subjected to a standardised capture-and-restraint protocol (Wingfield et al., 1992)—30 min in a bucket (20 L) freshly filled with seawater from the capture site (Pankhurst, 2001)—to determine a stress-induced cortisol response. After removal from the bucket of water, another ca. 0.08 ml sample of blood was obtained (as described above) for stress-induced measurement of cortisol only.

On return to the field station, blood samples were centrifuged (Sigma Centrifuge 1–14; <http://www.sigma-zentrifugen.de>) at 10,000 g for 5 min. The plasma was stored at -20°C until analysis. Plasma cortisol from both blood samples, and testosterone (T) and 11-ketotestosterone (11-KT) from the first blood sample were measured using EIA kits (Cortisol EIA Kit, No. 500360; T EIA Kit, No. 582701; 11-KT EIA Kit, No. 582751; Cayman Chemicals, SPI BIO, France) and a Beckman Coulter AD 340 Spectrophotometer at 405 nm as described in (Mills et al., 2010). The kits for cortisol and 11-KT have already been validated for this species (Mills et al., 2010; Beldade et al., 2017), but the kit for T was used after validation with parallel displacement of serially diluted plasma to the standard curve (SI 4, Table S3, Fig. S4).

Blood analyses were not carried out on samples collected from pairs subsequently excluded if they laid eggs or if the playbacks failed ($N = 6$ pairs); due to some difficulties in catching fish and the loss of some blood samples during centrifugation and defrosting, other samples were unavailable. Therefore, in Experiment 1, out of the targeted 20 anemonefish pairs, hormone samples were analysed from 28 fish (cortisol analysis, ambient-sound treatment: $N = 6$ females and $N = 9$ males; motorboat-noise treatment: $N = 6$ females and $N = 7$ males). In Experiment 2, out of the targeted 20 anemonefish pairs, hormone samples were available from 30 fish in total (cortisol analysis, ambient-sound treatment: $N = 7$ females and $N = 9$ males; motorboat-noise treatment: $N = 7$ females and $N = 7$ males). Blood samples were preferentially used to measure cortisol; if enough of the sample was available for further measures, 11-KT was measured, followed by T.

2.5. Data analysis

All analyses were performed in SPSS version 25. Sound-treatment differences in behaviour and hormones were analysed with Mann Whitney U and ANCOVA (body length as a covariate)

tests respectively. Relationships between behavioural and hormonal data were analysed using Spearman's rank correlations and linear regressions.

3. Results

3.1. Behavioural responses: Experiment 1

Anemonefish randomly allocated to the two sound treatments did not differ in their baseline behaviour. Considering the change in behaviour from silent playback to the first period of ambient-sound playback, there were no significant sound-treatment differences in hiding, distance from the anemone or aggression towards heterospecifics for either females or males (Table 1a).

Initial motorboat-noise playback caused strong behavioural responses in both females and males. Considering the change in behaviour from the first period of ambient-sound playback, both sexes exhibited a significant increase in hiding when exposed to motorboat-noise playback compared to ambient-sound playback (Table 1b; Figs. 2a and 3a). Both females and males also significantly decreased their mean distance from the anemone (Table 1b; Figs. 2b and 3b) and significantly increased their aggressive behaviour towards heterospecifics (Table 1b; Figs. 2c and 3c) in the motorboat treatment compared to the ambient treatment.

There was evidence of some short-term carry-over effect of motorboat-noise playback in females and males. Considering the difference between the first and second periods of ambient-sound playback, females exposed to motorboat-noise playback between these periods still showed significantly more hiding (Table 1c; Fig. 2d) and males were significantly closer to the anemone (Table 1c; Fig. 3e), with females showing a similar trend (Table 1c; Fig. 2c), compared to those in the ambient treatment. All other behaviours (male hiding and aggression in both sexes) showed no significant difference between sound treatments, indicating that they had returned to baseline levels once the motorboat-noise playback ceased (Table 1c; Figs. 2f, 3d and 3f).

There was no evidence for a short-term change in response to motorboat-noise playback with repeated intermittent exposure. During the third period of motorboat-noise playback, the behaviour of both females and males was similar to that in the first period of motorboat-noise playback, matching the lack of any difference in behavioural change in ambient-treatment fish over the same period (Table 1d; Figs. 2g–i, 3g–i).

3.2. Behavioural responses: Experiment 2

Despite a trend for females to increase hiding (Table 1e; Fig. 2j) and males to increase aggression (Table 1e; Fig. 3l) when exposed to motorboat-noise playback compared to ambient-sound playback, there was no evidence for a longer-term effect of intermittent motorboat-noise playback on baseline behaviour (i.e. when no motorboat noise was playing). There was no significant sound-treatment difference in either sex for any baseline behaviours after 48 h involving chronic daytime sound exposure (Table 1e; Fig. 2j–l, 3j–l).

Longer-term exposure to motorboat-noise playback did not alter the response to motorboat-noise playback. After 48 h including chronic daytime noise exposure, females and males still exhibited a significant increase in hiding, reduction in distance from the anemone and increase in aggressive behaviour towards heterospecifics when experiencing motorboat-noise playback compared to ambient-sound playback (Table 1f; Fig. 2m–o, 3m–o).

Table 1
Results of sound-treatment differences in behaviour analysed with Mann Whitney U tests. Results in bold indicate significant sound-treatment differences ($p \leq 0.05$).

Response	Behaviour	Female U	N	p	Male U	N	p
(a) Baseline behaviour (change from B4 to 1 st period in Experiment 1; Fig. S2)	Hiding	68.0	29	0.106	94.5	29	0.647
	Distance	91.0	29	0.541	83.5	29	0.348
	Aggression	83.0	29	0.280	78.0	29	0.164
(b) Initial effect of noise (change from 1 st to 2 nd period in Experiment 1; Fig. S2)	Hiding	53.0	29	0.023	39.0	29	0.004
	Distance	47.0	29	0.011	60.0	29	0.050
	Aggression	63.5	29	0.040	45.0	29	0.005
(c) Carry-over effect of noise (difference between 1 st and 3 rd periods in Experiment 1; Fig. S2)	Hiding	60.0	29	0.049	91.0	29	0.541
	Distance	66.0	29	0.089	60.0	29	0.050
	Aggression	92.5	29	0.488	93.0	29	0.535
(d) Short-term change in initial effect to noise (difference between 2 nd and 6 th periods in Experiment 1; Fig. S2)	Hiding	87.5	29	0.445	97.0	29	0.727
	Distance	103.0	29	0.930	71.5	29	0.144
	Aggression	93.5	29	0.574	80.0	29	0.221
(e) Longer-term effect of noise (m th period in Experiment 2; Fig. S2)	Hiding	15.0	16	0.081	30.0	16	0.874
	Distance	31.0	16	0.958	29.0	16	0.791
	Aggression	29.0	16	0.747	24.0	16	0.333
(f) Effect of noise after longer-term exposure (difference between m th and n th periods in Experiment 2; Fig. S2)	Hiding	12.5	16	0.044	8.0	16	0.013
	Distance	4.0	16	0.004	6.0	16	0.007
	Aggression	11.0	16	0.021	11.0	16	0.031

3.3. Hormonal responses

Cortisol levels were affected by longer-term, but not short-term, noise exposure. In Experiment 1, baseline circulating levels of cortisol following 30 min sound exposure did not differ between sound treatments for either sex (ANCOVA, female: $F_{1,10} = 0.334$, $p = 0.577$, Fig. 4a; male: $F_{1,14} = 0.446$, $p = 0.516$, Fig. 4b). After a standardised capture-and-restraint protocol, circulating levels of stress-induced cortisol were significantly higher than baseline levels (Table S4), but did not differ significantly between sound treatments for either sex (female: $F_{1,10} = 0.141$, $p = 0.716$, Fig. 4a; male: $F_{1,14} = 0.191$, $p = 0.669$, Fig. 4b). However, anemonefish of both sexes that were exposed to motorboat-noise playback over a period of 48 h (Experiment 2) had significantly higher baseline cortisol levels than ambient-treatment individuals (female: $F_{1,12} = 7.776$, $p = 0.018$, Fig. 4c; male: $F_{1,14} = 9.335$, $p = 0.009$, Fig. 4d). Moreover, although cortisol levels significantly increased after the standardised capture-and-restraint protocol for females exposed to ambient-sound playback for 48 h (repeated-measures ANOVA: $F_{1,6} = 6.326$, $p = 0.046$, Fig. 4c), this protocol caused a significant decrease in the cortisol levels of females exposed to longer-term motorboat-noise playback (female: $F_{1,4} = 77.875$, $p = 0.001$, Fig. 4c). For males, there was also a trend for increased cortisol levels after the standardised capture-and-restraint protocol to ambient-sound playback after 48 h ($F_{1,8} = 4.458$, $p = 0.068$, Fig. 4d) but, despite a decrease in cortisol levels of motorboat-treatment fish following standardised capture-and-restraint, this was not significant ($F_{1,5} = 0.765$, $p = 0.422$, Fig. 4d). As such, circulating levels of stress-induced cortisol did not differ significantly between sound treatments for either sex (ANCOVA, female: $F_{1,12} = 2.212$, $p = 0.165$, Fig. 4c; male: $F_{1,14} = 0.046$, $p = 0.833$, Fig. 4d).

In both experiments, sound treatment influenced male, but not female, testosterone (T) levels. In Experiment 1, T levels were significantly higher in males exposed to motorboat-noise playback

compared to ambient-sound playback (ANCOVA: $F_{1,9} = 26.714$, $p = 0.001$; Fig. 5a). There was a similar trend in females, but the difference between sound treatments was not significant ($F_{1,8} = 4.533$, $p = 0.071$; Fig. 5a). In Experiment 2, T levels were still significantly higher in males exposed to motorboat-noise playback over 48 h compared to those experiencing longer-term ambient-sound playback ($F_{1,11} = 11.730$, $p = 0.006$; Fig. 5b). As with the short-term experiment, there was no significant sound-treatment difference in female T levels ($F_{1,6} = 1.350$, $p = 0.298$; Fig. 5b).

In both experiments, there were sound-treatment differences in 11-ketotestosterone (11-KT) levels. 11-KT levels were significantly higher in males (ANCOVA: $F_{1,13} = 6.297$, $p = 0.027$, Fig. 5c), but not females ($F_{1,7} = 1.467$, $p = 0.271$, Fig. 5c), exposed to short-term motorboat-noise playback compared to ambient-sound playback. In Experiment 2, 11-KT levels were significantly higher in anemonefish of both sexes that were exposed to longer-term motorboat-noise playback compared to ambient-sound controls (male: $F_{1,15} = 7.188$, $p = 0.018$; female: $F_{1,9} = 20.544$, $p = 0.002$, Fig. 5d).

3.4. Linking behaviour and hormones

In Experiment 1, only female distance from the anemone was significantly related to baseline cortisol levels (Regression: $F_{1,9} = 5.861$, $p = 0.036$, Fig. 6a). Neither male distance from the anemone, nor hiding or aggression in either sex, were related to baseline cortisol, 11-KT or T levels (Tables S5a–c).

In Experiment 2, hiding increased with increasing baseline concentrations of cortisol for both sexes (Regression, male: $F_{1,11} = 5.360$, $p = 0.041$, Fig. 6b; female: $F_{1,9} = 5.560$, $p = 0.043$). For both sexes, aggression increased with increasing 11-KT levels (Spearman's rank correlation: male: $r_s = 0.609$, $N = 15$, $p = 0.016$; female: $r_s = 0.814$, $N = 10$, $p = 0.004$) and increasing T levels (male: $r_s = 0.646$, $N = 11$, $p = 0.032$, Fig. 6c; female: $r_s = 0.926$, $N = 7$, $p = 0.003$).

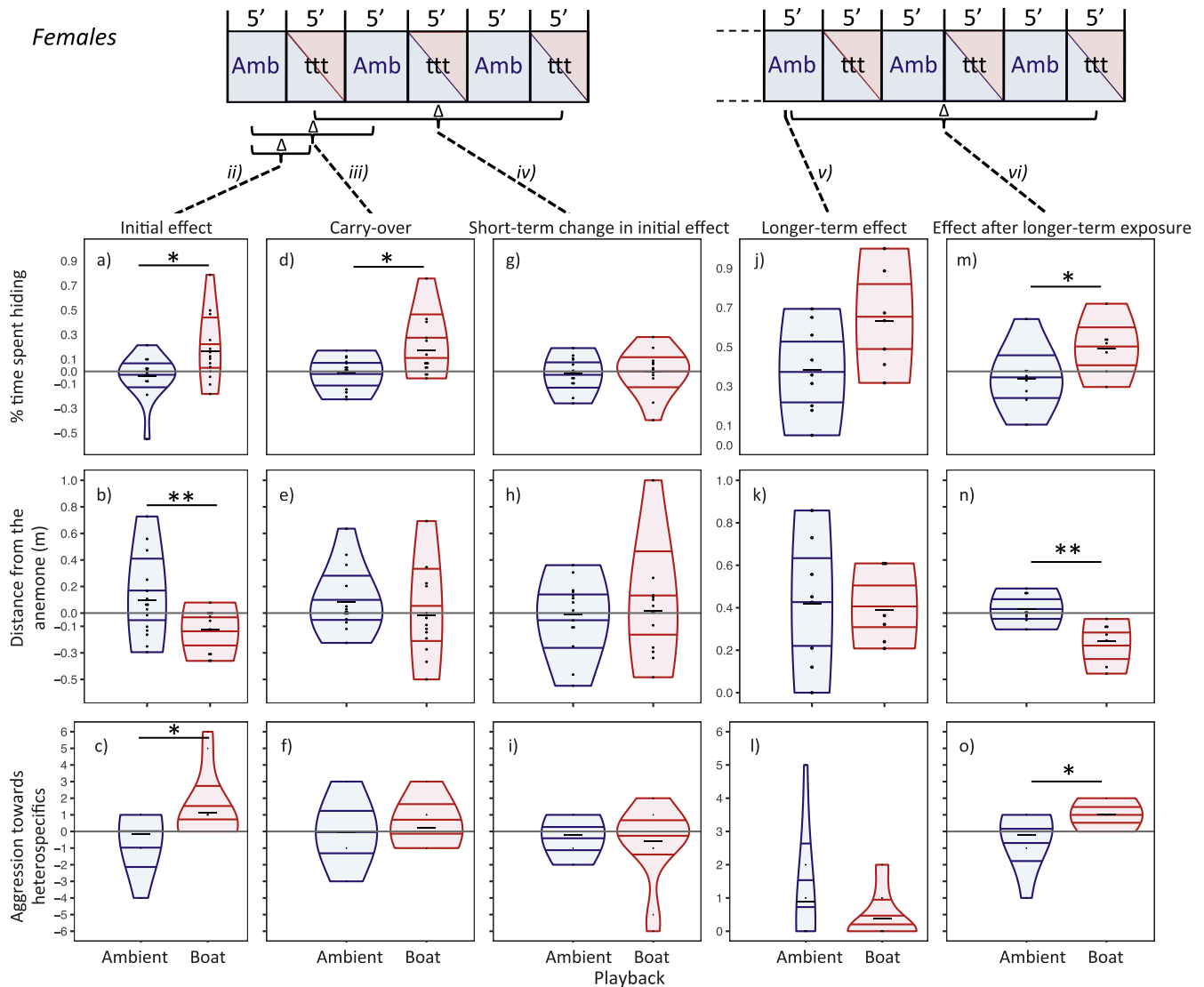


Fig. 2. Short and longer-term behavioural responses of female orange-fin anemonefish *Amphiprion chrysopterus* exposed to either ambient-reef-sound playback only (blue) or ambient-reef-sound playback with intermittent motorboat-noise playback (red). Shown as differences in behaviours (% time spent hiding in anemone; distance moved from the anemone and aggression towards heterospecifics). a–c) *Initial effect* (ii) of motorboat-noise playback—the change from the first ambient-sound period to the next 5 min period, which was either another ambient-sound period or a motorboat-noise period depending on sound treatment (difference from 0 suggests an initial effect). d–f) *Potential for carry-over* (iii) of motorboat-noise playback immediately after it ceased—sound-treatment differences between the first and second ambient-sound periods in Experiment 1 (similarity with a,b,c indicates presence of carry-over). g–i) *Short-term change in initial effect* (iv) to motorboat-noise playback with repeated intermittent exposure—differences between the first and third motorboat-noise playback periods and their equivalents in the ambient-sound treatment (difference from 0 suggests the presence of a short-term change in response from after first exposure). j–l) *Longer-term effect* (v) of intermittent motorboat-noise playback on baseline behaviour—behaviour during ambient-sound playback after 48 h exposure to either ambient sound only or ambient sound with intermittent motorboat noise (differences between red and blue data show differences between treatments). m–o) *Effect after longer-term exposure* (vi) of motorboat-noise exposure—differences between the first ambient-sound period and the final 5 min period, which was either another ambient-sound period or the third motorboat-noise period depending on sound treatment (difference from 0 suggests a longer-term, chronic, effect). Violin plots show the full distribution of the data with the inner part showing all sample points (dots), the median (bold line) and the interquartile range overlaid by the kernel density estimation. The width of the plot represents the number of observations for a given y value; a wider plot has more observations at that value.

4. Discussion

Free-living anemonefish exposed to intermittent motorboat-noise playback in the short-term (30 min) responded both behaviourally and hormonally. Male and female hiding and aggression increased, and both sexes moved less distance out of the anemone. There were no effects on cortisol levels, but male androgens (11-ketotestosterone (11-KT) and testosterone (T), not previously measured in response to motorboat noise), also increased. Some behaviours showed carry-over effects from 5 min of noise exposure, but there was no evidence for a change in response to subsequent motorboat-noise playback in the short-term (over 30 min).

Similarly, there was no evidence that longer-term exposure led to changes in response to noise: motorboat noise had an equivalent effect on male and female anemonefish behaviour and hormones after 48 h as on first exposure. However, whilst longer-term noise exposure did not affect baseline behaviour, it did lead to higher levels of circulating glucocorticoid (cortisol) in both sexes and higher T levels in males. Moreover, male and female stress-responses to an additional environmental challenge were impaired in fish exposed to longer-term motorboat noise. Circulating androgen levels correlated with aggression, while cortisol levels correlated with hiding, demonstrating for the first time in a wild population that androgen/glucocorticoid pathways are the

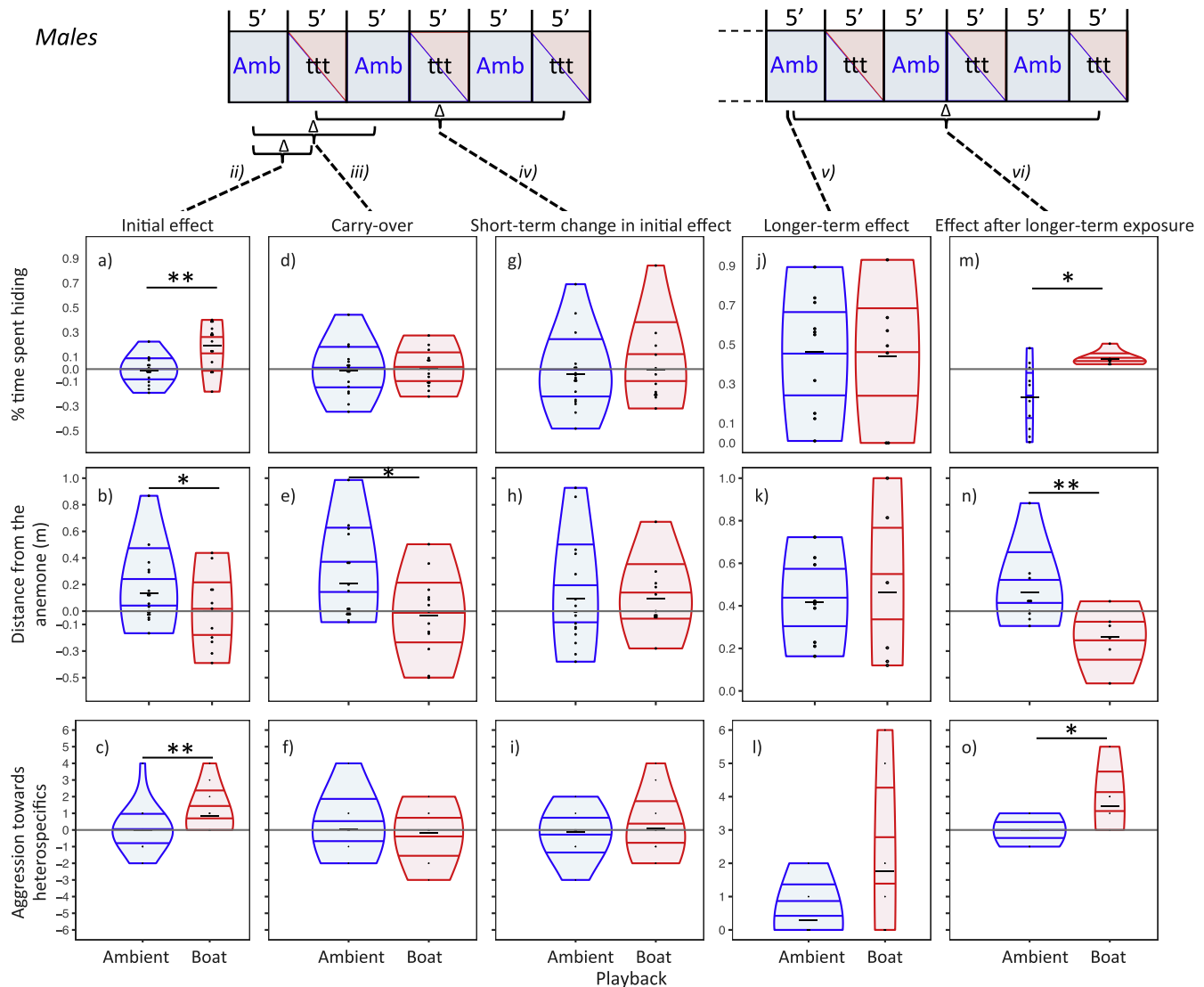


Fig. 3. Short- and longer-term behavioural responses of male orange-fin anemonefish, *Amphiprion chrysopterus*; same colour scheme and description as per Fig. 2.

plausible proximate mechanisms driving behavioural responses to anthropogenic noise.

4.1. Behavioural effects

Anemonefish responded to both short- and longer-term exposure to motorboat-noise playback with increased levels of territorial aggression to heterospecifics at or near their host anemone. Elevated levels of aggression would increase energetic expenditure and, potentially, resting metabolic rates (Norin et al., 2018). Anemonefish would therefore need to compensate for such elevated energy demands with increased rates of foraging, but motorboat noise also increased levels of hiding within the anemone and reduced the distance moved out of the anemone, providing little opportunity for such compensation. Similarly, *Gobius cruentatus* spent more time inside its shelter when exposed to ferry- and boat-noise playbacks (Picciulin et al., 2010), and juvenile damselfish moved less distance during boat-noise exposure (Holmes et al., 2017). We also found carry-over effects for hiding and distance moved out of the anemone for the 5 min period after a motorboat passage, similar to carry-over effects of cheating on clients by

cleanerfish exposed to motorboat noise (Nedelec et al., 2017a); there may be a latency period greater than 5 min before these behaviours diminish. Such residual effects of motorboat noise would further compromise the ability of anemonefish to compensate with elevated foraging. To exacerbate the problem, previous studies have shown that motorboat noise can negatively affect foraging itself, in terms of time spent, efficiency and the number of foraging errors (Purser & Radford, 2011; Wale et al., 2013; Voellmy et al., 2014).

In principle, organisms exposed to repeated or chronic environmental stressors may show rapid tolerance in the short-term and/or habituation in the longer-term (Biro, 2012), which would lessen the potential impact of a stressor. For instance, juvenile damselfish exposed to repeated anthropogenic noise showed evidence of behavioural desensitisation in under 20 min (Holmes et al., 2017) and there was a reduction in physiological responses to noise by European seabass after 12 weeks of noise exposure (Radford et al., 2016a). However, anemonefish in our experiments did not show any indication of a reduction in response either in the short-term or following 2 days of noise exposure, which was also the case for the behaviour of spiny chromis after 12 days of noise

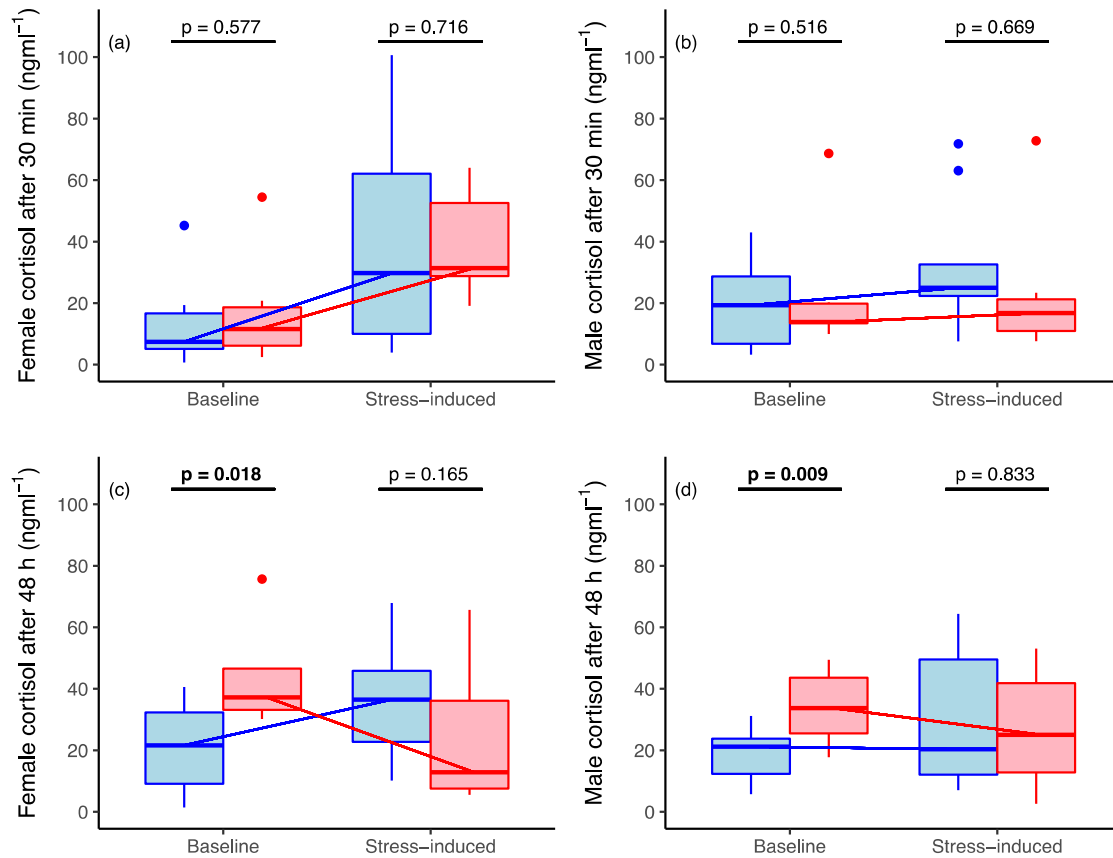


Fig. 4. Mean (\pm SE) initial (baseline) cortisol concentration (ng ml⁻¹) and stress-induced cortisol concentration (ng ml⁻¹) responses of orange-fin anemonefish, *Amphiprion chrysopterus* exposed to either ambient-reef-sound playback (blue) or motorboat-noise playback (red) for either 30 min for (a) females and (b) males or for 48 h for (c) females and (d) males.

exposure (Nedelec et al., 2017b). While it would be necessary to run the experiment for many weeks to be sure that no reduction in response eventually occurs with repeated exposure, our experiments provide no indication that this might be the case. As such, motorboat noise will potentially have detrimental impacts on the growth and survival of site-attached species, such as anemonefish, that do not have the option to move away from an environmental stressor.

4.2. Glucocorticoid response pathways

Short-term (30 min) exposure to motorboat noise did not seem to influence circulating levels of cortisol in anemonefish. It is possible that the noise-exposure regime (3 x 5 min of motorboat passage plus 3 x 5 min of ambient-reef sound) may not have been sufficiently stressful to elevate cortisol levels. Whilst exposure to boat noise elevated cortisol levels in goldfish within 10 min (Smith et al., 2004) and within 30 min in three other fish species (Wysocki et al., 2006), these were laboratory studies with different noise playbacks, and different species may require significantly more time or stronger noise stressors to activate a cortisol response (Wikelski et al., 1999). Despite the lack of a discernible effect on cortisol levels, we did find a significant effect of motorboat noise on behaviour usually associated with elevated stress (i.e. more hiding and less distance moved from shelter). These rapid behavioural changes may therefore have occurred via acute neuroendocrine regulation through neural cortisol synthesis (Pradhan et al., 2014).

Despite no short-term response, baseline cortisol levels of anemonefish were elevated following longer-term (48 h) noise exposure. These elevated levels are indicative of chronic stress due to the activation of the HPI axis. Chronic stress is common in a range of taxa in response to anthropogenic noise (birds: Hayward et al., 2011; Blickley et al., 2012b; mammals: Creel et al., 2002; amphibians: Tennessen et al., 2014; Kaiser et al., 2015; fishes: Wysocki et al., 2006). During motorboat-noise exposure, anemonefish increased hiding in their host anemone, which increased with increasing levels of cortisol. Other studies have found increased hiding and less distance moved during boat-noise exposure (Picciulin et al., 2010; Holmes et al., 2017), but this is the first time that this behaviour has been correlated with the stress response. Furthermore, the effect of motorboat noise on baseline stress levels is comparable with other challenges experienced during the life of anemonefish, including their response to a 3 month warming-induced bleaching event (Beldade et al., 2017), indicating the significance of noise as a pollutant. Finally, baseline cortisol levels were elevated in the longer-term experiment providing no evidence of a reduction in response of the HPI axis to motorboat noise, in agreement with findings in roach after an 11 day motorboat noise exposure, but contrary to those from Eurasian perch (Johansson et al., 2016). These results suggest that the HPI axis did not habituate to motorboat noise.

The stress-induced cortisol response of anemonefish exposed to longer-term motorboat noise declined, rather than increased, from baseline cortisol levels. This down-regulation of a stress-induced

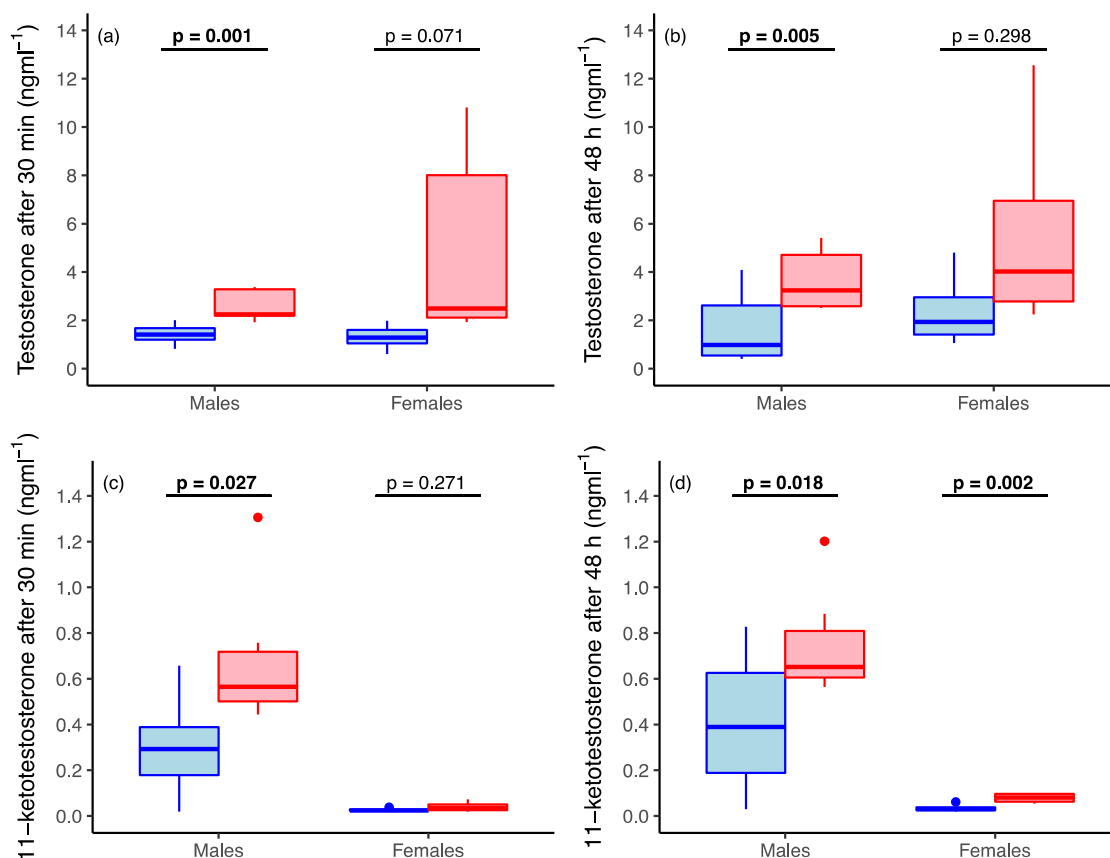


Fig. 5. Short- and longer-term hormonal responses of orange-fin anemonefish, *Amphiprion chrysopterus* exposed to either ambient-reef-sound playback (blue) or motorboat-noise playback (red). Boxplots showing median (—), 25th–75th quartiles, 95% confidence intervals and outliers (•): (a) male plasma testosterone concentration (ng ml^{-1}); (b) female plasma testosterone concentration (ng ml^{-1}); (c) male plasma 11-ketotestosterone concentration (ng ml^{-1}); and (d) male plasma 11-ketotestosterone concentration (ng ml^{-1}).

response is often a direct consequence of chronic stress prior to capture, indicating that the HPA (or HPI) axis was compromised (Hontela et al., 1992; Norris et al., 1999). Similar down-regulation of stress-induced responses have previously been found in fish exposed to other environmental stressors, including effluents (Pottinger et al., 2013) and contaminants (Koakoski et al., 2014). Chronic stress has been shown to damage either the HPA (or HPI) axis at the hypothalamic regulatory level, with a reduced release of arginine vasotocin that down-regulates cortisol responses (Rich & Romero, 2005), or fish interrenal tissue directly causing a down-regulation of adrenocorticotrophic/GC hormone receptors resulting in a reduced secretion of cortisol (Brodeur et al., 1997; Koakoski et al., 2014). Such impairments of the HPA (or HPI) axis impact the ability to cope with environmental challenges (Sneddon et al., 2016) and the lack of a GC response to an additional stressor has fitness consequences, as demonstrated by higher mortality due to predation in chronically stressed fish shortly after a stressful event (Mesa, 1994).

4.3. Androgen response pathways

The elevated aggression towards heterospecifics observed in the short-term exposure to motorboat noise was not correlated with either steroid (11-KT and T) and may be due to the time delay from the end of behavioural observations through to capture, preparation and eventual blood sampling. In the future, it would be informative to measure androgen levels more rapidly and on a finer

scale, every 5 min during motorboat noise exposure, to determine the pathways involved. Furthermore, the lack of carry-over effects indicates swiftly changing levels of aggression over our short-term (30 min) experimental period, so the mechanism involved needs to respond rapidly to environmental conditions. The timeframe is too short for either the production of androgens to be explained by the classical hypothalamic–pituitary–gonadal (HPG) axis (Oliveira, 2009) or for the behavioural changes to be explained by DNA transcriptional changes (slow genomic actions) of steroids (Moore & Evans, 1999). Alternatively, 11-KT and T could have been rapidly produced in the brain, either synthesised *de novo* by the brain–gonadal pathway (Oliveira, 2009) or converted from steroids via steroidogenic enzymes present in brain cells (Perry & Grober, 2003; Pradhan et al., 2014) and the neurosteroids entered circulation. Subsequently, the rapidly changing behavioural responses to motorboat noise (aggression changing within 5 min) were likely due to fast non-genomic actions of steroids, via rapid activation of cell membrane receptors and intracellular signalling pathways that modulate neural circuits controlling behaviour (Oliveira, 2009; Thomas & Pang, 2012). Rapid behavioural responses (within 1 s–2 min) to motorboat noise found in scallops, *Pecten fumatus* (Day et al., 2017), three-spined sticklebacks, *Gasterosteus aculeatus* (Purser & Radford, 2011), damselfish, *Pomacentrus amboinensis* (Holmes et al., 2017), and European eels, *Anguilla anguilla* and seabass, *Dicentrarchus labrax* (Bruintjes et al., 2016) may have been driven by similar non-genomic pathways. Circulating levels of 11-KT and T were similar in both the short- and longer-term

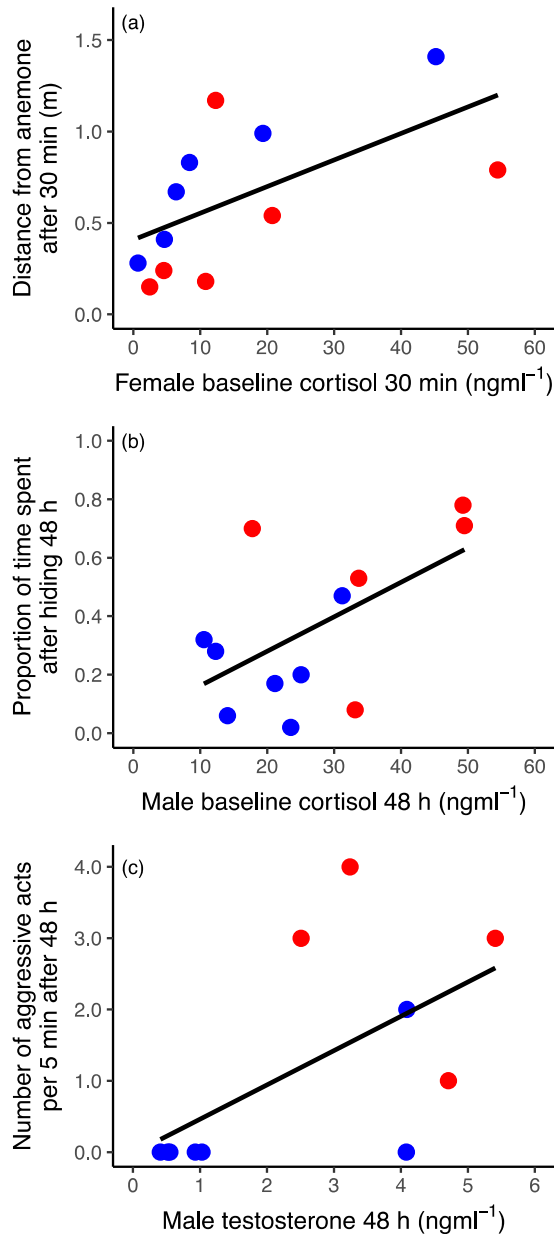


Fig. 6. Relationship between glucocorticoid and steroid hormones on behaviour. Relationship between: (a) initial (baseline) cortisol concentration (ng ml⁻¹) in females and distance from anemones (m) after 30 min (regression: $y = 0.409 + 0.015x$, $r^2 = 0.37$); (b) initial (baseline) cortisol concentration (ng ml⁻¹) in males and proportion of time spent hiding in anemone after 48 h (regression: $y = 0.036 + 0.012x$, $r^2 = 0.31$); and (c) testosterone concentration (ng ml⁻¹) in males and number of aggressive acts after 48 h (Spearman's rank correlation: $r_s = 0.926$, $p = 0.003$).

experiments, providing no evidence of habituation in androgen responses to motorboat noise. Our results from the longer-term exposure experiment suggest a classic activational role of circulating androgens on behavioural responses. The genomic actions of steroids occur mostly after 1 h to several days after entering the cell nucleus and mediating changes in DNA transcription of steroid-sensitive genes that modulate behaviour (Moore & Evans, 1999) and our 48 h experiment fits well within this timeframe. Accordingly, aggression is correlated with both 11-KT and T in males and females after our longer-term noise-exposure period.

Motorboat noise may be similar to natural sounds, such as

predator calls, that are construed as a threat, elevate androgen levels and elicit behavioural responses (Tyack et al., 2011). However, when the motorboat has passed, sounds can no longer be misconstrued, androgen levels rapidly decline and androgen-driven behavioural responses return to normal levels (i.e. no carry-over). Whilst we found carry-over effects for cortisol-driven behaviours (hiding and distance moved out of the anemone), we did not for the androgen-driven behavioural response aggression, neither did other studies measuring parental-care and nestling-begging behaviour (Naguib et al., 2013). Our results indicate that the presence of a behavioural carry-over effect to motorboat-noise playback varies with the type of behaviour in question and which hormonal pathway governs the behaviour.

5. Conclusions

The neuroendocrine system is arguably the most important physiological mechanism underlying survival in our changing world (Wingfield, 2008). Activation of this system modifies entire suites of physiological, behavioural and cellular traits and we have demonstrated in a wild fish population that motorboat-noise playback triggers GC and androgen responses with concomitant impacts on behaviour. In light of the impacts on behaviour, their potential impacts on fitness, and the impacts of anthropogenic noise previously found on reproduction (Picciulin et al., 2010; Naguib et al., 2013) and survival (Simpson et al., 2016; Day et al., 2017; Nedelec et al., 2017a), it would be advantageous from an evolutionary perspective if GC and androgen responses adjusted to anthropogenic noise. The neuroendocrine system can become modified, or even permanently altered, as a function of the stressors experienced during either an individual's development (developmental plasticity), their lifetime (phenotypic plasticity) or their parent's lifetime (epigenetic changes) (Angelier & Wingfield, 2013; Bjilmsma & Loeschcke, 2005). However, there are little data on whether such modifications are adaptive (but see Tennessen et al., 2018). It is critical to understand if and how species/populations will cope with anthropogenic noise, and this study demonstrates the significant role played by the neuroendocrine system.

Understanding the proximate mechanisms driving behavioural changes may prove a useful tool in regulating anthropogenic stressors and mitigating their impacts. Detailed knowledge of the initiation, carry-over, recovery and habituation times of hormonal responses, such as those tested here, would enable us to predict the duration and/or interval times of stressor exposure that allow individuals to return to normal behaviour. Such knowledge could be particularly used in Marine Protected Areas or during sensitive periods of the life-cycle (e.g. reproduction and/or spawning). Furthermore, hormonal assays could be used as measures of impact to compare the value of different mitigation approaches (different boat engines, propeller design, buffer zones, etc.). In short, due to their important role in modifying physiological, behavioural and cellular traits, hormonal responses represent an underemployed tool for the rapid and accurate measurement of the impacts of anthropogenic stressors, including noise.

Notes

Ethical approval from Centre National de la Recherche Scientifique (Permit Number: 006725).

Declaration of competing interest

The authors declare no conflicting interests.

CRedit authorship contribution statement

Suzanne C. Mills: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Ricardo Beldade:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Funding acquisition. **Laura Henry:** Investigation, Writing - review & editing. **David Laverty:** Investigation, Writing - review & editing. **Sophie L. Nedelec:** Methodology, Formal analysis, Investigation, Writing - review & editing, Visualization. **Stephen D. Simpson:** Conceptualization, Methodology, Formal analysis, Resources, Writing - review & editing, Project administration, Funding acquisition. **Andrew N. Radford:** Conceptualization, Methodology, Formal analysis, Resources, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2020.114250>.

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