



# **Anemonefish Behaviour in a Changing World**

Submitted by Isla Marguerite Hawthorne Hely  
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Signature:

A handwritten signature in black ink that reads "I. Hely". The signature is written in a cursive style with a long horizontal stroke at the end.

## Abstract

Coral reefs are among the most biodiverse ecosystems in the oceans, harbouring 25% of all known marine species. Amongst the hubbub of biogenic noise, fish contribute significantly to the cacophony of reef sounds. However, with 25% of the human population living on the world's coastlines, coral reefs are experiencing unprecedented change. Using wild orange-fin anemonefish (*Amphiprion chrysopterus*) female–male adult pairs (Moorea, French Polynesia), this thesis aims to: 1) explore the *in situ* natural vocal repertoire of *A. chrysopterus*, and 2) test for impacts of light pollution on diurnal behaviour. Previous studies assessing anemonefish acoustic communication and the influence of artificial light at night (ALAN) on anemonefish behaviour are predominantly laboratory-based. For the first time, by assessing *in situ* vocal behaviour using a single-pulse analytical approach, this research found new layers of complexity in *Amphiprion* communication. Orange-fin anemonefish were found to: i) infrequently engage in acoustic communication unless exposed to a 'rival' conspecific, ii) show sex-based differentiation in pulse peak frequency, iii) vary substantially in pulse peak frequency within and between pulses and iv) diverge from the previously-reported relationship between fish size and pulse frequency. Following *in situ* exposure to IMMEDIATE (one night) and PROLONGED (14–35 nights) exposure to ALAN (~12–26 lux), subsequent diurnal behaviour of female anemonefish was not significantly influenced. In contrast, male behaviour was significantly altered, but only following long-term exposure. This research deepens our understanding of anemonefish vocal behaviour and demonstrates that male and female anemonefish have differing responses to the stressor of light pollution; important in developing mitigation for fishes in sex-structured social groups. Overall, this thesis showcases anemonefish as a valuable model species for *in situ* fieldwork, with the methodologies developed here proposed as being easily-adapted to assess the effects of other stressors on fish behaviour in this rapidly-changing world.

## Acknowledgements

Isn't it ironic how I have managed to (finally) complete my Master's thesis in the midst of a global pandemic and many hardships, yet when I come to write this acknowledgements paragraph I reach peak struggle! I struggle, because, to put into words how thankful I am to Prof. Steve Simpson and Prof. Andy Radford, seems a task at which I could only ever fail. You are both truly singular humans; deeply intelligent, highly supportive, and innately kind. I have been blessed to be under your supervision. I wish to also thank Dr Lucy Hawkes, a formidable scientist and brilliant secondary supervisor. This Masters would not have been possible without Dr Suzanne Mills and Dr Ricardo Beldade, key collaborators and simply put, great friends. You saved me and my Masters, and without you both I wouldn't be at this point today. All this would also not have been possible without the fundamental help and friendship of '*L'équipe poisson-clown*': Thibaut Roost, Jo-Ann Schies, Jules Schligler, Emma Weschke & Ben Williams. Thank you for keeping me alive in the field and putting up with my, *franchement*, questionable French. I would also like to thank Exeter Marine, for providing me with a research grant, fundamental in making this research possible and the University of Exeter for allowing me to undertake such a fulfilling Masters by Research. I have learnt so much and furthered myself more than I believed possible, so for this opportunity I am most grateful. I would also like to extend my thanks to my examiners for their invaluable feedback on my thesis.

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## Author's Declaration

Both data chapters in this thesis describe my contribution to a collaborative research project with Dr Suzanne Mills (Associate Professor at EPHE) that is to be submitted for publication as part of a wider study on orange-fin anemonefish. I confirm that all material that is not my own has been identified and that any material that has been previously submitted and approved for the award of a degree by this or any other University has been acknowledged.



Chapter 1:

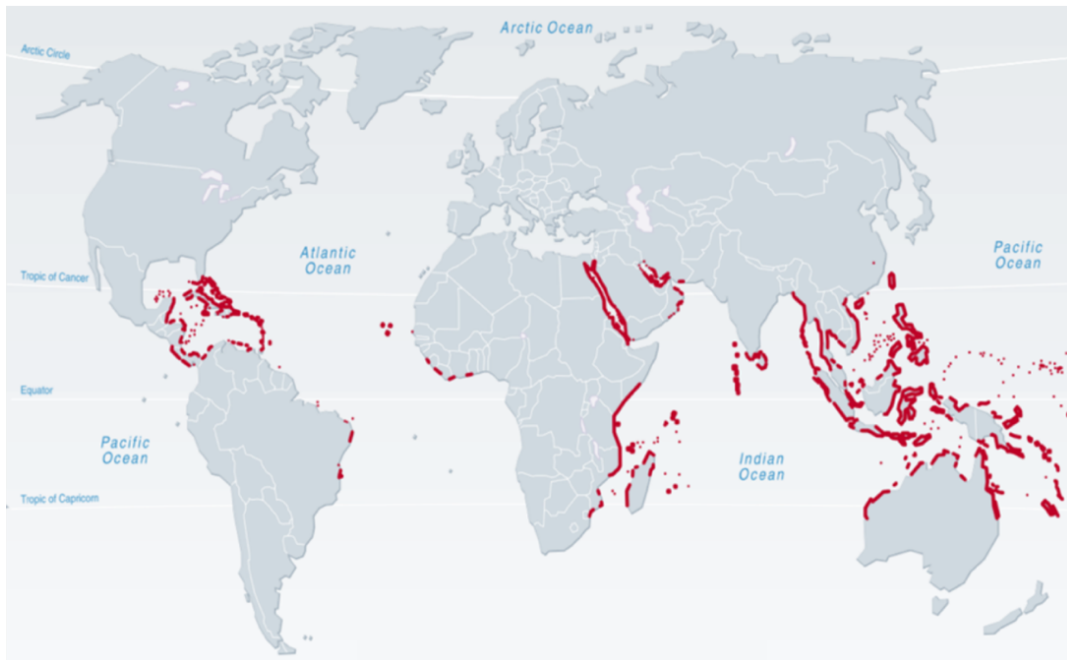
General Introduction

In this introductory chapter, I present coral reefs as ecosystems where a mosaic of species interactions and behaviours occur. I begin by describing visual and vocal communication in coral reef fishes and highlight the current knowledge gap in understanding coral reef fish communication *in situ*. I then highlight the unprecedented rates of change in these fragile ecosystems, discussing the myriad global and local anthropogenic stressors present in these important coastal areas. In doing so, I identify light pollution as a significantly understudied marine stressor. Finally, I introduce the orange-fin anemonefish (*Amphiprion chrysopterus*) as a model species to study *in situ* fish vocal communication and to assess the impacts of stressors on behaviour in the wild.

## 1.1 Coral Reefs: Cities by the Shore

Oceans cover 71% of the Earth's surface and provide 99% of the world's habitable space. By acting as both a carbon sink, absorbing ~50% of atmospheric carbon dioxide, and an oxygen source, releasing 50% of the global oxygen produced by photosynthesis (Balamurugan et al., 2014), oceans are fundamental for the existence of life on Earth. The high level of primary production in marine ecosystems supports oceanic food webs, with a broad diversity of organisms ranging from some of the smallest animals on our planet (e.g., microzooplankton; 20–200  $\mu\text{m}$  long) to the largest (the blue whale, *Balaenoptera musculus*; ~30 m long). There is no greater density of biodiversity than that found on coral reefs, the most biologically diverse marine habitats in the world, estimated to house between 600,000 and 9 million species (Reaka-Kudla, 1997; Plaisance et al., 2011). Straddling the tropics, coral reefs harbour ~25% of all described marine species (Plaisance et al., 2011), yet account for just 0.1% of the world's oceans (Spalding et al., 2001; Fig. 1.1). Coral reefs exist in a variety of forms, including fringing reefs, barrier reefs, atolls and isolated patch reefs (Stoddart, 1969; Hopley, 2011). The diverse range of organisms living on reefs provide key ecosystem services including, but not limited to, protection from storms, fisheries

(reefs act as key nursery and foraging grounds) and provision of raw materials (Moberg & Folke, 1999; Cesar, 2003; Grafeld et al., 2017). The annual value of these services has been estimated at \$30 billion (Ferrario et al., 2014).



**Figure 1.1.** A world map highlighting the distribution of the world's coral reefs (excluding deep-water temperate reefs) (obtained from UNEP World Conservation Monitoring Centre).

### 1.1.1 Fish Behaviour

Unparalleled species richness on coral reefs means that they harbour a complex mosaic of biological interactions (Ferrario *et al.* 2014). Reef organisms interact with individuals of the same species (conspecific interactions) and of differing species (heterospecific interactions). Coral reef organisms also interact with physical reef structures for rest, feeding opportunities, spawning/reproduction, finding shelter/refuge and provision of cleaning stations (Paterson, 1998; Fréon & Dagorn, 2000; Barreiros et al., 2002). This introduction will focus solely on the interactions of coral reef fishes.

Many thousands of fish species depend on coral reefs for some or all of their life (Slabbekoorn et al., 2010; Fig. 1.2). Despite the availability of a wide variety of microhabitats (Slabbekoorn et al., 2010), the high diversity of coral reef fishes means that species overlap significantly in relation to habitats and functions (Mouillot et al., 2014), resulting in a high likelihood of interaction. To survive on reefs, fish must feed and forage (Mouillot et al., 2014), whilst avoiding predators (Holbrook & Schmitt, 2002), but they also engage in social interactions with conspecifics and heterospecifics. Common conspecific interactions are predominantly related to reproduction (i.e., mating and courting behaviours) and aggression (i.e., exerting dominance/displaying submission, threatening, territoriality), with both visual and vocal communication often a key component.



**Figure 1.2.** Snapshot of a reef in the Maldives, Indian Ocean showcasing an array of fish species from various families, including Labridae, Pomacentridae and Balistidae. Image: Isla Hely.

### 1.1.2 Fish Communication

Communication allow animals to share information and concurs fitness benefits for both signaller and receiver (Moberg & Folke 1999; Nedelec et al., 2015).

Visual and acoustic displays are two well-established methods for communication in fishes.

#### 1.1.2.1 Visual Communication

Visual communication on reefs is well documented, with visual displays being especially important for reproduction. For example, male coral trout (*Plectropomus leopardus*) present distinct courtship colourations and swimming displays which culminate in mass spawning aggregations, where trout density can increase 12.5-fold (Samoilys & Squire, 1994). During a single mating, hamlets (simultaneous hermaphrodites in the genus *Hypoplectrus*) display egg-trading behaviours (Fischer 1980), where individuals alternate between offering eggs to be fertilised and fertilising the eggs of their partner. This can occur up to four times during one mating and involves the male curving its body around the female, harbouring the upward-floating eggs. Other reef fishes reproduce in nests in specific locations on reefs where visual communication is also key. For example, territorial male blue damselfish (*Chrysiptera cyanea*) display courtship behaviours where the male swiftly approaches the female, fanning his tail fins and displaying lead swims (where he swims towards the nest in a directed movement; Fischer 1980). In turn, compliant females also display their fins and form a solicitation posture where they bend their body into a sigmoidal shape (Gronell, 1989). Post-spawning, female *C. cyanea* have minimal involvement in the nest, with males fanning and guarding eggs until they hatch (Thresher & Moyert, 1983; Gronell, 1989).

Visual assessment of size and of secondary sex characteristics are important in aggressive interactions in coral reef fish species. For example, male giant bumphead parrotfish (*Bolbometopon muricatum*)—the largest teleost fish on coral reefs—visually size each other up before engaging in ‘headbutting’ behaviours, where the males rapidly collide with their cephalic humps (a bulbous

protrusion from the skull). Much like in cetartiodactyl species (even-toed ungulates) such as red deer (*Cervus elaphus*), these male–male aggressive collisions are associated with the establishment of dominance and mating territories (Lusseau, 2003).

Many reef fish are site-attached and so commonly defend their territories with aggressive behaviours including biting and chasing (Thresher & Moyert, 1983; Gronell, 1989; Holbrook & Schmitt, 2005). For example, the dusky damselfish (*Stegastes dosropunicans*) tends and defends a ‘farm’ of epilithic algae (Myrberg, 1972), while anemonefish form a symbiotic relationship with a host anemone which they defend from invertivores in return for the anemone acting as a refuge from piscivores (Fricke, 1979; Fautin & Allen, 1992; Beldade et al., 2016). Aggressive interactions in nest-guarding male *Chrysiptera cyanea* usually involve visual displays where males erect their dorsal, pelvic and anal fins and chase each other, with agonistic interactions lasting from just a few seconds up to >30 minutes (Thresher & Moyert, 1983; Gronell, 1989).

#### 1.1.2.2 Acoustic Communication

Due to the high molecular density of water, sound travels five times faster underwater than in air (Slabbekoorn et al., 2010). This also means sound propagates over much greater distances than can visual and olfactory cues, which are limited by the rapid attenuation of light underwater and the displacement of currents respectively. Previously presented as a silent world by Jacques Yves Cousteau in his 1956 documentary *Le Monde du Silence*, advancement in technology (e.g., underwater microphones called hydrophones) now enables capture and characterisation of high-resolution sound enabling myriad underwater sounds to be examined by a growing field of bioacousticians. Due to this advancement, different coastal habitat types have been characterised as having specific ambient sounds (Slabbekoorn et al., 2010), with healthy coral reefs being particular biodiversity hotspots (McWilliam et al., 2017). The cumulation of sound produced by animals in coral reef communities creates

distinct choruses that change from day to night, and with varying seasonal patterns (McCauley, 2012; McWilliam et al., 2017). Major sources of sound on reefs include the background crackle of snapping shrimp (*Alpheus heterochaelis*) (McCauley 2012; McWilliam et al., 2017), as well as the pops and grunts of coral reef fishes (Myrberg et al., 1993; Kaplan et al., 2015).

Sound production in fishes is highly diverse across taxa, with fish possessing the most varied range of sound-production apparatuses among vertebrates (Ladich & Fine, 2006). This broad range is, in part, due to the independent evolution of fish acoustic mechanisms along different lineages, unlike other homologous sonic mechanisms such as those found in birds and mammals (syrinx and larynx respectively; Ladich & Fine, 2006). The two main mechanisms of sound production in fishes are: (1) the drumming of the swim bladder via contraction of the surrounding sonic muscles, as seen in fishes such as drums and croakers (Family Sciaenidae); and (2) the stridulation of hard skeletal parts or teeth, such as the rapid closure of the pharyngeal jaw and teeth, as seen in damselfish, including clown anemonefish (Family Pomacentridae; Bass & McKibben, 2003; Lobel et al, 2010).

Fishes vary considerably in their hearing abilities (Popper & Fay, 2011), but the majority detect sound in two main ways. Some species can use only peripheral structures along the lateral line of the body, called neuromasts, to detect particle motion caused by sound displacing water at frequencies lower than 100 Hz (Slabbekoorn et al., 2010; Hawkins, 2011). Other fishes can also detect sound via otoliths located near the brain. Otoliths are calcium carbonate structures which vibrate in response to the particle motion of sound waves at frequencies ranging from hundreds to thousands of hertz (Slabbekoorn et al., 2010). This vibration, in turn, moves surrounding cilia which enervates neuromasts and is interpreted by the brain as sound. In some taxa, the swimbladder—an important teleost-specific buoyancy organ—can further

increase hearing sensitivity, dependent on the presence and proximity of this organ to the otoliths (Montgomery et al., 2006).

It is becoming increasingly apparent that many coral reef fish communicate acoustically, with the biophony from fish often dominating the reef soundscape (Moberg & Folke, 1999; Nedelec et al., 2015). Of the 179 fish families present in coral reefs, 48 are known to be vocal, with the frequencies of these vocalisations ranging from below 100 Hz to up to 8 kHz (Lobel et al. 2010). Vocalisations are associated with myriad coral reef fish behaviours. Fish sound production is heightened during the breeding season (Bass & McKibben, 2003), highlighting the importance of sound production for reproduction. On reefs, 15 coral reef fish families have been shown to vocalise during breeding (Bass & McKibben, 2003), with males being the predominant vocalisers, aiming to attract females to nearby nest sites (Lobel et al., 2010). Additionally, sound production has been observed during aggressive encounters, particularly in association with submissive, chasing, fleeing, distress and threatening behaviours (Ladich, 1997). Fish commonly vocalise when defending their territories from potential invaders and predators. This is most frequently seen in site-attached species, such as nocturnal soldier and squirrel fishes (family Holocentridae; commonly found in refuges under coral heads during the day), that produce grunts and staccato vocalisations to deter, for example, invading moray eels (Winn et al., 1964; Salmon, 1967). Similarly, damselfishes (family Pomacentridae) frequently vocalise to defend their territories from both heterospecifics and conspecifics (Winn et al., 1964; Salmon, 1967; Santiago & Castro, 1997). Acoustic communication is therefore a key component of aggressive interactions, allowing for information to be transferred efficiently without escalation to energetically costly physical disputes (Ladich & Myrberg, 2006). Additionally, acoustic communication is vital in shoaling fish, where nocturnal vocalisations act as contact calls aiding in group cohesion; for example, for bigeyes (*Pempheris adspersa*), a nocturnal planktivorous reef fish species (Van Oosterom et al., 2016).



Considering (1) fishes represent the largest, most diverse group of vertebrates (Ravi & Venkatesh, 2008), (2) mammal and avian auditory mechanisms are modifications of the more basic functions found in early fish (Ravi & Venkatesh 2008), and (3) the importance sound plays in fish life histories and survival, with all studied fish thus far being able to detect sound (Fay & Popper 2000), it is surprising that research into fish acoustic communication remains rather limited (Bassi et al. 2021).

## 1.2 Stress on Reefs

As the 'cities of the ocean', coral reefs are characterised as biodiversity hotspots for species and biological interactions. However, the world is changing at an unprecedented rate (Slabbekoorn et al., 2010). Owing to their shallow, proximal, coastal locations, coral reefs are often situated in close proximity to humans, with numerous associated stressors. The high level of biodiversity on reefs could act as insurance against environmental perturbations and/or ecosystem collapse through high levels of functional redundancy, with stabilising assemblages that can sustain specific ecological functions (the 'portfolio effect'; Thibaut et al., 2012). However, exposure to increasingly intense anthropogenic pressures has led to the stability of ocean ecosystems being put at risk (Faulkner et al., 2018), with 58% of the world's reefs now threatened by human activities (Spalding et al., 2001; Faulkner et al., 2018).

Anthropogenic stressors on coral reefs can be classified into global and local stressors (Spalding et al., 2001). The influence of global stressors is felt oceanwide and requires international collaboration in order to minimise and mitigate against associated negative effects (Brown et al., 2013). Global stressors relating to climate change include ocean warming and ocean acidification (Sabine et al., 2004; Hoegh-Guldberg et al., 2007). The increased atmospheric concentration of carbon dioxide has resulted in approximately 25%

of all CO<sub>2</sub> produced from anthropogenic sources being absorbed by our oceans per year (Canadell et al., 2007). This has resulted in: (1) an increase in the average temperature of the world's oceans of 0.74°C, (2) an increase in average sea level of 17 cm and (3) an increase in ocean acidity by the reduction of pH by 0.1 unit (Canadell et al., 2007; IPCC, 2007). On the other hand, local stressors are limited to the immediate region in which they occur and can be more easily manipulated via direct management (Canadell et al., 2007; IPCC 2007). Local stressors include overfishing, eutrophication, and noise and light pollution (Putland et al., 2017; Friedlander et al., 2018). Stressors, both global and local, can act (and interact) in myriad ways, influencing the environment and sensory ecology of marine species oceanwide (Putland et al., 2017).

#### 1.2.1 Global Stressors

Coral reefs are predicted to be particularly susceptible to the negative effects of climate change considering tropical reef species have lived for millions of years in a relatively stable environment and are therefore adapted to a limited thermal and acidic range (Checkley et al., 2009; Simpson et al., 2011a). The raised concentration of oceanic carbonic acid as a consequence of increased atmospheric CO<sub>2</sub> hinders carbonate accretion in calcifying species ranging from organisms at the foundation of the food chain (e.g., planktonic coccolithophores; (Putland et al., 2017)) to habitat engineers, such as reef-building scleractinian corals (Kleypas et al., 1999; Raven, 2005). When carbonate levels drop to extreme low levels (~200 mmol kg<sup>-1</sup> seawater), reef-building corals can physically erode (Kleypas et al., 1999; Raven, 2005). Projections of the combined effects of ocean acidification and ocean warming suggest that by the year 2100, coral reefs globally will struggle to maintain net positive carbonate production (Cornwall et al., 2021). The loss of corals results in reduced habitat complexity (Cornwall *et al.* 2021), which negatively influences reef-associated invertebrates and fishes that normally benefit from the refugia provided by healthy complex reefs (Jones et al., 2004). Ocean acidification can also influence non-calcifying organisms,

with negative effects on survival, calcification, growth and reproduction (Jones et al., 2004).

Ocean acidification has many negative effects on juvenile teleost fish, including survival (Baumann et al., 2012), behaviour (Munday et al., 2009a, 2009b; Ferrari et al., 2011), metabolism (Wittmann & Pörtner, 2013) and development (Munday et al., 2012). Due to a greater ability to regulate ions, adult fish are believed to be somewhat more resilient than earlier life stages to ocean acidification (Claiborne et al., 2002; Evans et al., 2005; Melzner et al., 2009), while eggs and larvae are more susceptible to negative effects (Ishimatsu et al., 2005; Brauner, 2008; Simpson et al., 2011a). Additionally, ocean acidification has been shown to alter significantly the growth of the inner-ear otoliths of fish, which can alter the auditory sense (Checkley et al., 2009; Simpson et al., 2011b). The unprecedented rise in sea surface temperature (Hoegh-Guldberg et al., 2007) is causing coral bleaching and metabolic stress in a range of organisms (Hughes et al., 2003; Cheung et al., 2013). For example, two species of coral reef fishes, *Ostorhinchus doederleini* and *O. cyanosoma*, displayed a reduced capacity for aerobic activity (oxygen consumption) when exposed to extreme water temperatures (29°C; Munday et al., 2009a). Interestingly, *O. doederleini* and *O. cyanosoma* were similarly influenced by CO<sub>2</sub>-acidified water (pH 7.8 and ~1000 ppm CO<sub>2</sub>), highlighting how stressors can have additive negative effects (Munday et al., 2009a).

### 1.2.2 Local Stressors

The proximal location of humans living close to coral reefs is not surprising considering the many ecosystem services reefs provide. For example, fisheries associated with coral reefs are a vital source of income and food for hundreds of millions of people worldwide (UNEP, 2004). However, overfishing is a widespread problem in tropical regions (UNEP, 2004), involving the capture of fish at a rate

greater than the fish can reproduce. This has resulted in precipitous population density declines of coral reef fishes (Halpern et al., 2008; Muñoz et al., 2012). Removal of key species that provide specific functions on reefs can be catastrophic for reef ecosystems. For example, the removal of herbivorous fishes (e.g., parrotfishes) that feed on algae results in significant algal cover increase (Shantz et al., 2020). Increased algal cover can decrease coral growth rates by 37%, with overfishing aiding in the prevalence of algal-dominated reefs, causing scientists to refer to this phenomena as the 'slippery slope to slime' (Thurber et al., 2012). Additionally, overfishing can lead to a change in fish behaviour, with exploited fish avoiding humans throughout their range, as seen in the case of the highly valuable 75 kg humphead parrotfish *Bolbometopon muricatum* (Munoz et al., 2012).

Eutrophication — the influx of excess anthropogenic nutrients (e.g., nitrogen and phosphorous) — also has devastating effects, particularly on coral growth, reproduction and survivorship (Barton, 2002), as well as intensifying coral disease (D'Angelo & Wiedenmann, 2014; Shantz & Burkepile, 2014). Boosted by nutrient pollution, algae can outcompete coral (Zaneveld et al., 2016) resulting in the increasing frequency of harmful algal blooms (Zaneveld et al., 2016), along with associated fish mass mortality (Heisler et al., 2008; Paerl & Huisman, 2008).

Anthropogenic noise is another type of local stressor and comes in many forms, from ship and motorboat noise to seismic air guns and pile driving. It overlaps with the hearing and vocalisation ranges of a multitude of marine organisms (Boyd et al., 2011; Moore et al., 2012; Tricas & Boyle, 2014; Neenan et al., 2016) and is contributing to the increase in ambient sound levels oceanwide (McDonald et al., 2006; Hildebrand, 2009). Anthropogenic noise affects many marine organisms, from cetaceans to zooplankton (McCauley et al., 2017; van Ginkel et al., 2017). On coral reefs, motorboat noise is a pervasive form of noise pollution and has negative ecological and physiological effects on coral reef organisms. For instance, motorboat noise has been found to inhibit predator-

learning and avoidance (Simpson et al., 2016; Ferrari et al., 2018), alter embryonic development (Jain-Schlaepfer et al., 2018), decrease parental care and offspring survival (Nedelec et al., 2017), and disrupt larval orientation and settlement behaviour (Nedelec et al., 2017). It has also been shown to cause elevated metabolic rate, heart rate and cortisol levels (Remage-Healey et al., 2006; Wysocki et al., 2006; Simpson et al., 2016; Jain-Schlaepfer et al., 2018; Fakan & McCormick, 2019), which are indicators of a stress response (Barton, 2002).

Negative effects of local stressors can exacerbate those of global stressors. For example, Cembella et al. (2002) found that nitrogen enrichment interacts with the negative effects caused by ocean warming (e.g., heat stress) to influence coral bleaching across >10,000 coral colonies. The negative effect of local stressors can reduce the ecological resilience—defined as the ability of an ecosystem to return to a baseline state following a perturbation or disturbance—of coral reef ecosystems to global stressors (Hughes et al., 2003). Considering the predicted increase in the frequency of perturbations and disturbance associated with global warming and climate change (e.g., increased extreme weather events (IPCC 2007) and bleaching events (Hughes et al., 2017)), there is a fundamental need to understand the effects of these small-scale local stressors. In doing so, mitigation of local stressors may aid in resilience to global stressors in coral reef ecosystem.

In summary, local stressors such as eutrophication, fishing pressure and noise pollution can all have substantial impacts on coral reef fish assemblages (Friedlander et al. 2017). However, there is another local stressor, both pervasive and intense in some coral reef ecosystems and yet highly understudied – light pollution.

### 1.2.3 Light Pollution: A Novel Stressor

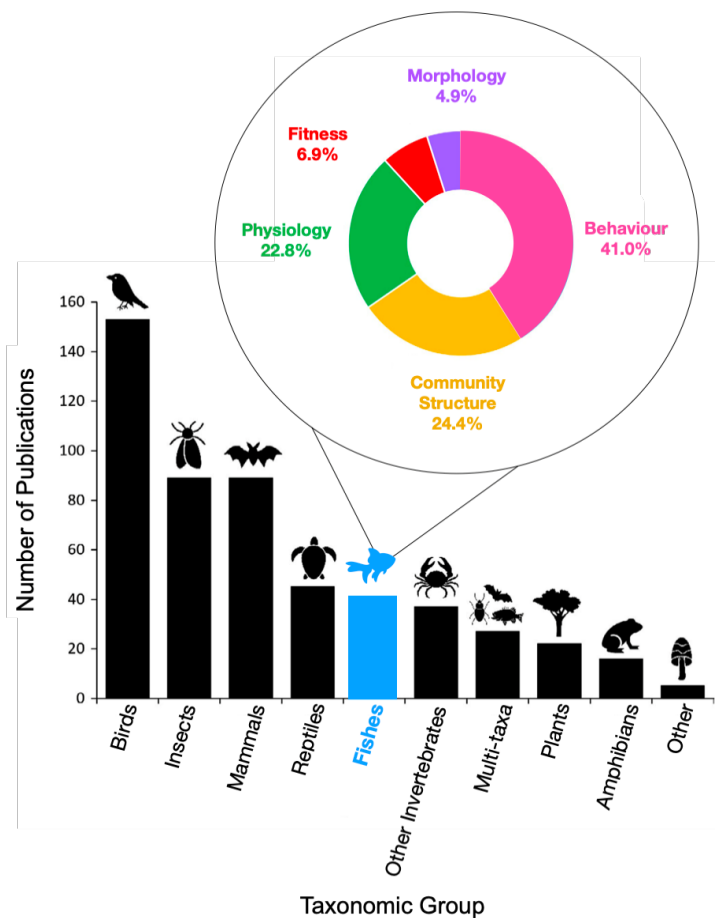
Artificial light covers much of the globe and is rapidly expanding, with a predicted 2.2% increase in brightness per year (Falchi et al., 2016) and shifting spectral characteristics (Hölker et al., 2010; Kyba et al., 2017). Since the formation of the Earth 4.5 billion years ago, light from celestial bodies, such as the sun, moon and stars, has bathed the Earth, fluctuating naturally from day to night (Leech & Johnsen, 2022). Before the widespread availability of gas and electricity lighting dating from the late 1800s (Longcore et al., 2010), natural variation in light provided a reliable environmental cue used by myriad organisms. Throughout evolutionary time, light has played a fundamental role in orchestrating biological activity, resulting in highly preserved biological clocks and circadian and circannual rhythms that regulate and coordinate a range of behaviours and physiology in animals (Gaston et al., 2013; Brüning et al., 2018). These highly evolved systems are affected by extended day-length cues from artificial light at night (ALAN) that pervade all but the most isolated ecosystems.

Studies on the effects of ALAN have focused predominantly on terrestrial species, with 59% of publications on light pollution impacts having this focus, compared to 20% aquatic and 21% mixed systems (Bassi et al., 2021). Birds are particularly well-studied, with significant effects of ALAN found for migration (Leech & Johnsen 2022), song (Kempnaers et al., 2010; Longcore, 2010), timing of reproduction and egg-laying (Havlin, 1964; Lack, 1965; De Molenaar et al., 2006), testosterone production and gonad development (De Molenaar et al., 2006), and mortality (Harvie-Brown & Cordeaux, 1880). ALAN has negative effects on a wide range of other taxa, including amphibians (Wise, 2007; Perry et al., 2008), bats (Stone et al., 2009), moths (Van Geffen et al., 2014), insects (Rich & Longcore, 2006), mammals and reptiles (Rich & Longcore 2006).

In general, research on the impacts of ALAN on wildlife has seen an increase in recent years (Gaston et al., 2015; Spoelstra et al., 2015), yet there are some key gaps in our understanding (Hölker et al., 2010; Van Grunsven et al., 2017). For instance, marine ecosystems and organisms are comparatively less studied compared to terrestrial ecosystems, with papers published on fishes showing a decline each year since 2016 (reaching a maximum of just 6 papers published in a single year; Bassi et al. 2021). This is surprising considering 25% of the world population lives within coastal regions, where artificial light can be most pervasive (Elvidge et al., 1997; Cinzano et al., 2001; Small & Nicholls, 2003). With a growing global human population, more extensive stretches of the coastline are predicted to become increasingly exposed to artificial light (Depledge et al., 2010). Light pollution in these regions can emanate from hotels, roads, streetlights, boardwalks, piers, fisheries and ships (Davies et al., 2016), with light from these structures permeating into the atmosphere to produce a secondary source of ALAN called skyglow (Kyba et al., 2011). Effects of ALAN on marine ecosystems has been documented for sea turtles (Tuxbury & Salmon, 2005; Bourgeois et al., 2008; Mazor et al., 2013) and marine birds (Black, 2005; Montevecchi, 2006; Merkel & Johansen, 2011), as well as a limited range of other fauna. Considering fishes make up the largest, most diverse group of vertebrates, with >35,000 species (Ravi & Venkatesh, 2008), it is notable that fishes thus far have been underrepresented (Fig. 1.3).

To date, the majority of studies assessing the effects of ALAN on fishes have been conducted in the laboratory, or when carried out in the wild, predominantly in freshwater ecosystems (Becker et al., 2013; Foster et al., 2016; Nelson et al., 2021; but see: Bolton et al., 2017; Schligler et al., 2021). Nonetheless, this work has highlighted the effects of ALAN on behaviours such as swimming activity (Talanda et al., 2018; Pulgar et al., 2019; Lin et al., 2021), foraging (Talanda et al., 2018; Czarnecka et al., 2019), movement, migration and dispersal (Riley et al., 2012, 2013) and risk-taking (Sabet et al., 2016). Effects of ALAN on physiology include reduced melatonin (Brüning et al., 2016; Khan et al., 2018), altered cortisol secretion (Newman et al., 2015; Szekeres et al., 2017) and

reduced expression of reproductive hormones (Newman et al., 2015; Szekeres *et al.* 2017). Finally, effects of ALAN beyond behaviour and physiology include altered abundance and community structure (Keenan et al., 2007; Barker & Cowan, 2018), decreased fitness (Brüning et al., 2011) and altered morphology (Grace & Taylor, 2017; Fig. 1.3). Although valuable for understanding the effects of ALAN in isolation, laboratory studies are limited in their capacity to mirror natural environments, especially important environmental and ecological processes that occur *in situ*, including predator–prey interactions, variable habitat availability and environmental stochasticity (Talanda et al. 2018; Bassi et al. 2021). Additionally, assessment of impacts of the atmospheric scattering of light and resulting skyglow is not possible in a laboratory (Gaston et al., 2015; Kyba *et al.*, 2017). This is particularly poignant considering skyglow can prevail over hundreds of kilometres (Kyba & Hölker 2013), with terrain having a limited blocking effect, unlike with direct illumination (Aubé, 2015; Gaston et al., 2015). With research into the effects of ALAN on fishes in recent decline (Bassi et al., 2021), it is vital to obtain a more holistic understanding of *in situ* threats of ALAN



on coral reef fishes to aid in fisheries management and enable targeted conservation (Bassi et al., 2021).

**Figure 1.3.** The number of publications from 1965 to 2021 in the primary literature that have focused on ALAN, partitioned into taxonomic group. The associated pie chart depicts the percentage of research topics covered in publications focusing on the effects of artificial light at night on fish. Figure sourced and adapted from Bassi et al. (2021).



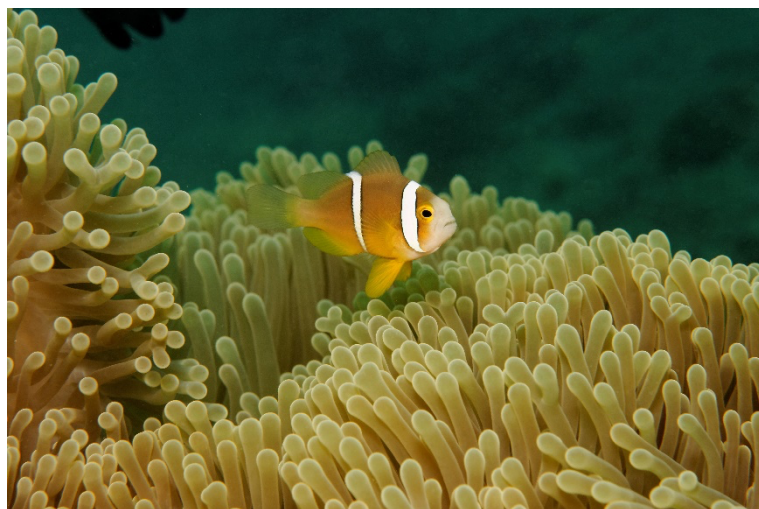
### 1.3 Anemonefish: A Model Coral Reef Fish

Owing to the shallow, easily accessible and clear-water characteristics of coral reefs, as well as the diverse range of soniferous fishes that live there, these ecosystems are prime locations for the study of fish acoustic communication and *in situ* exploration of the effect of environmental stressors. Pomacentrids are among the most studied coral reef fishes and many are site-attached, displaying varied social interactions with specific visual and vocal displays (Grace & Taylor, 2017). Pomacentrids are also among the most vocal of fishes, producing a wide range of sounds in different behavioural contexts (Parmentier et al., 2007). The family Pomacentridae is characterised as being one of the most successful radiations of coral reef fishes (Bellwood & Wainwright, 2002; Frédérick et al., 2013), encompassing a total of 394 described species (Eschmeyer, 2014). Anemonefish (subfamily Amphiprioninae) constitute 30 of these species and have been widely studied, with their baseline life-history traits (e.g., reproductive and social behaviours; Buston, 2003a, 2003b, 2004a, 2004b; Buston & Elith, 2011; Eschmeyer, 2014) and anemone–fish symbioses (Mebs, 1994; Balamurugan et al., 2014) both well understood. Previous *in situ* research has highlighted that adult anemonefish are vulnerable to noise (Mills et al., 2021) and juvenile anemonefish are vulnerable to artificial light at night (Schligler et al., 2021). Wong and Buston (2013) previously highlighted how habitat-specialist fishes, such as fish from the family Pomacentridae, are particularly tractable species, albeit for the testing of theories on social evolution. Therefore, I selected anemonefish as previously suggested biologically and logistically appropriate and tractable coral reef fish model species, enabling complementary *in situ* assessments of vocal communication and effects of ALAN. I focused on the orange-fin anemonefish, *Amphiprion chrysopterus* (Pomacentridae).

### 1.3.1 Anemone–anemonefish Symbioses

The symbiosis between anemonefish and their host anemone is an iconic marine mutualism. Orange-fin anemonefish live within the tentacles of their host anemone, *Heteractis magnifica* (Fig 1.4). They form an obligate relationship with the fish protecting the host from predators whilst simultaneously being protected from predation by the venomous tentacles of the anemone (Moyer & Bell, 1976). Direct contact with the anemone significantly redefines the anemonefish microbiome, allowing anemonefish to live harmoniously and sting-free with their host (Pratte et al., 2018). Anemonefish are aggressive towards their own predators and predators of their eggs, and are also aggressive towards predators of the host anemone (e.g., butterflyfish) and with conspecifics and heterospecifics living in the same anemone (e.g., *Dascyllus trimaculatus*; Mariscal, 1970; Fricke, 1974, 1975; Ross, 1978; Moyer, 1980; Godwin & Fautin, 1992). When anemonefish are not present, anemones can be consumed within 24 hours (Fautin & Allen, 1997), highlighting the importance of this symbiosis. In addition to aggression, the waste of anemonefish has nutritional value, sustaining the anemone and aiding in its growth and asexual reproduction (Meyer & Schultz, 1985a, 1985b; Holbrook & Schmitt, 2005). More recently, anemonefish have

been found to benefit from nutrients and carbon obtained from the host anemone too, highlighting a potential chemical recycling loop within this symbiosis (Verde et al., 2015). Recent work has highlighted how anemone size can influence anemonefish growth,



**Figure 1.4.** A juvenile orange-fin anemonefish (*A. chrysopterus*) situated above its host anemone, the magnificent sea anemone (*H. magnifica*). Image: Frederick Zuberer.

with juvenile *Amphiprion percula* relocated to large anemones showing significantly increased growth compared to those relocated to small anemones (Rueger et al., 2021). Previous work has also shown that the behaviour of different *Amphiprion* species can affect the growth of the host anemone, with less bold species being an important limiter of anemone growth (Schmiege et al., 2017).

### 1.3.2 Social Grouping & Reproduction

Orange-fin anemonefish live in groups of unrelated individuals (Buston et al., 2007), with adults mainly living in breeding pairs with varying numbers of associated non-breeding juveniles and recruits who have a negligible effect on adult fitness (Fautin & Allen, 1992, 1997; Buston, 2003a, 2004a). A highly social species, orange-fin anemonefish has a size-dominance hierarchy with the female being the largest, most dominant fish in the group, followed by the adult male, with a hierarchy of juveniles ranked by size (Fricke, 1979; Buston, 2003a; Buston & Cant, 2006). Non-breeders form a queue for breeding position (Buston, 2004a), with the separated size differences of individuals in each rank meaning that if an individual is removed (due to mortality or migration), then the individual of the rank directly below can ascend rank, and grows in size (Buston, 2003a, 2003b, 2004b; Mitchell, 2005). Orange-fin anemonefish are protandrous hermaphrodites, meaning that if the largest female dies (or migrates from the host anemone), the largest male in the group changes sex, adopting the role of the most dominant fish in the group (Fricke, 1979; Parmentier et al., 2009).

The monogamous adult breeding pair spawn year-round, breeding up to twice per lunar month; the majority of fish spawn around the full moon with some able to spawn a second time around the new moon (Mitchell, 2003; Beldade et al., 2016). The pair can produce and raise offspring tens to hundreds of times (Buston 2003a; Buston and Elith 2011), and display differing levels of involvement in parental care (Moyer & Bell, 1976). Female orange-fin anemonefish invest

significant energy on egg production and laying, producing clutches of 500–3000 eggs, with the size of the brood being positively related to the number of eggs that successfully mature to hatching (Barbasch et al., 2020). Males act as the primary egg-carers, displaying particular preparatory behaviours such as cleaning an area on the underside of the anemone in readiness for the female (Ross, 1978; Beldade et al., 2016). Once the female has laid her eggs, the male fertilises them and continues to guard and defend them from potential predators until they hatch, whilst removing any algae and dead eggs from the brood (Beldade et al. 2016). Males also fan the eggs, a behaviour thought to be associated with increasing the flow of oxygen and improving gaseous exchange across the egg–seawater boundary (Moyer & Bell, 1976; Beldade et al., 2016; Barbasch & Buston, 2018). Male care-giving behaviours are positively related to egg survival (Barbasch et al., 2021), and continue until the eggs hatch on (usually) the seventh day.

### 1.3.3 Vocal Behaviour

Pomacentrids are highly soniferous fishes, and 27 *Amphiprion* species have been found to produce sound (Colleye et al., 2011). *Amphiprion* species produce sound via stridulation of the pharyngeal jaw (Parmentier et al., 2007): under tension, the cerato-mandibular (*c-md*) ligament pulls the jaw teeth together, with the subsequent collision producing a sharp ‘pop’ (Parmentier et al., 2007; Olivier et al., 2015). This ligament is important not just for vocalisation, but also for feeding and biting (Olivier et al., 2015), which are two of the main axes of diversification in vertebrates (Streelman & Danley, 2003; Olivier et al., 2015).

*Amphiprion* species vocalise under a range of ecological and social contexts; for example, when interacting aggressively with other fish and during reproductive behaviours (Schneider, 1964; Takemura, 1983; Parmentier et al., 2005; Ladich, 2015). In conjunction with vocal communication, female and male *A. chrysopterus* protect the host anemone by displaying agonistic behaviours

such as chasing behaviours (Colleye et al., 2009); females are frequently more aggressive and territorial than males. Due to the size-dominance hierarchy, females exert dominance by aggressively charging and chasing conspecific males, with larger males charging and chasing subordinate males (Moyer & Bell, 1976); these agonistic behaviours are all frequently coupled with vocalisations (Colleye et al., 2009). Subordinates can display submission via head-shaking movements as a response to aggressive displays from more dominant fish which are frequently associated with submissive sounds, such as chirping (Colleye & Parmentier, 2012).

To date, the majority of research into anemonefish acoustic communication has been performed in the laboratory, with a focus on agonistic interactions (Parmentier et al., 2007; Colleye et al., 2009; Colleye et al., 2012; Colleye & Parmentier, 2012). To the best of my knowledge, no studies have aimed to assess the *in situ* vocal repertoire and associated behaviours of orange-fin anemonefish.

#### 1.4 Thesis Aims

In light of the increased intensity of stressors on coral reefs (Spalding et al., 2001; Faulkner et al., 2018), greater understanding of natural baseline behaviours and communication in coral reef fishes is required for a full assessment of the effects of both global and local stressors in a changing world.

**Chapter 2** provides a deeper dive into the vocal repertoire of the site-attached coral reef anemonefish, orange-fin anemonefish. A first ethogram of *in situ* vocal behaviour was produced by exposing female–male fish pairs to mirrors (i.e., ‘perceived intruders’) on the northern reefs of Mo’orea, French Polynesia. Using *in situ* video-captured vocalisations, I compared metrics of vocalisations dependent on sex and behaviour using a single-pulse analytical approach. I found greater complexities in individual vocalisation trains than previously described,

and explore whether size-based variation in frequency found in lab conditions also occurs *in situ*.

**Chapter 3** provides the first *in situ* assessment of indirect effects of ALAN on adult diurnal behaviour in orange-fin anemonefish. Assessing both short- and long-term effects of ALAN on four behaviours (sociability, hiding, aggression, vocalisation), I tested whether adult daytime behaviour was influenced by ~1 night's exposure to ALAN, and also 14–35 nights exposure to ALAN. I hypothesised that there would be a negative influence on behaviour, with any negative effect either (i) diminishing over long-term exposure (i.e., acclimation) or (ii) accumulating over time (i.e., sensitisation).

**Chapter 4** provides a general discussion, linking the findings from both data chapters and highlighting implications of the findings and adaptability of the methodologies.

## Chapter 2:

A deeper dive into anemonefish  
vocalisation and behaviour in their  
natural environment

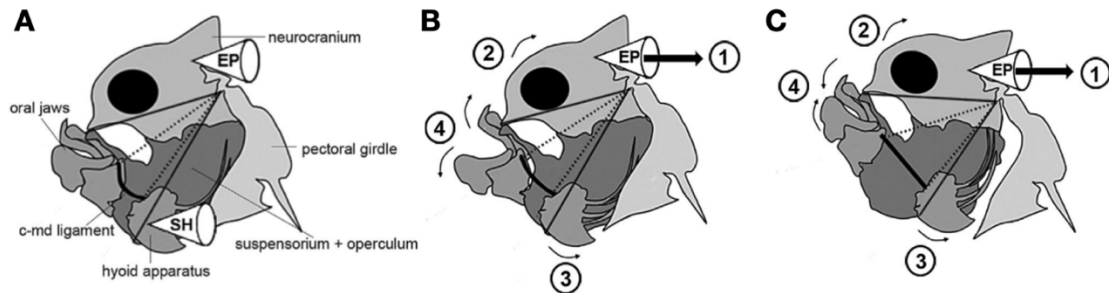
## 2.1 Introduction

Coral reefs are some of the most diverse ecosystems on the planet, harbouring ~25% of all marine species yet covering less than 1% of the Earth's surface (Plaisance et al., 2011). In these highly diverse systems, coral reef species live in close proximity to each other, resulting in myriad conspecific and heterospecific interactions and social behaviours, including acoustic communication (Lobel et al., 2010). Reefs are therefore home to an array of soniferous species (Tricas & Boyle, 2009, 2014), with the cacophony of sound emanating from these ecologically important ecosystems being vital for the recruitment of larvae and the replenishment of local fish stocks (Simpson et al., 2004, 2005, 2008, 2011).

More than 300 species of fish inhabiting coral reefs are known to produce sound for communication (Slabbekoorn et al. 2010; Tricas & Boyle, 2014). Fish vocalisations propagate well, since sound travels five times faster in water than in air ( $1500 \text{ ms}^{-1}$  versus  $300 \text{ ms}^{-1}$  respectively; Lobel et al., 2010) with little attenuation. It is therefore unsurprising that fish have a highly conserved auditory sense, highlighting the evolutionary importance and benefit of sensing sound (Lobel et al., 2010). Pomacentridae is a highly vocal family of fish, comprising damselfishes and anemonefishes. Anemonefishes (*Amphiprion* and *Premnas* species) are territorial, site-specific fish, that live in close-association with a symbiotic host anemone (Fricke, 1979; Fautin & Allen, 1992; Beldade et al., 2016). *Amphiprion* species are known to vocalise when displaying aggressive behaviours, emitting sequences of pulses ranging from one to 15 'pops' within a single vocalisation pulse train (Amorim, 1996). The frequencies of these pulses range from 370 to 900 Hz, although *Amphiprion* species can detect sounds between 75 and 1800 Hz (Parmentier et al., 2009). *Amphiprion* species produce sound by use of a sonic ligament, also referred to as the cerato-mandibular ligament (*c-md* ligament; Olivier et al., 2015). Under tension, the *c-md* ligament allows the rapid elevation of the lower jaw followed by a rapid lowering of the hyoid apparatus ( $23.8 \text{ m/s}^2$ ; Parmentier et al., 2007). This, in turn, causes the



jaw teeth to collide, with kinetic energy being converted to sonic energy with the jaws radiating the ‘pop’-like sound (Fig. 2.1). This phenomenon, in which bodily structures are rubbed and/or gnashed together, is called stridulation.



**Figure 2.1.** A detailed schematic representation of the sound-producing mechanism found within *Amphiprion* species. White cones represent two muscles: epaxial muscles (EP) and sternohyoideus muscles (SH). In (A) the fish is not moving, with the cerato-mandibular (*c-md*) ligament loose. Following a contraction of the EP (B1), the neurocranium is rotated (B2) which in turn rotates the hyoid apparatus (B3) resulting in the straining of the *c-md* ligament (B4). Further contraction of the EP (C1) causes more severe rotation of the neurocranium and hyoid apparatus (C2 and C3) resulting in the jaws coming together (C4). The hyoid bar (1) moves and stretches the sonic ligament (the *c-md* ligament) (2) causing the rapid closure of the jaw resulting in teeth collision and a resulting vocalisation ‘pop’. Dotted and black angles measure the relative movement of: (i) the two insertion points of the *c-md* ligament and (ii) the neurocranium and the hyoid apparatus respectively. Image obtained and adapted from Olivier et al. (2015).

Fish vocalisations are diverse, with individual calls having particular characteristics that are not only dependent on the species, but also morphological traits of the individual (Colleye et al., 2011). Calculating acoustic metrics of sound, such as dominant frequency and pulse duration, aids in characterising and quantifying specific vocalisations (Colleye et al., 2011; Parmentier et al., 2016). For example, previous studies have highlighted size-related variation in acoustic metrics for a range of *Amphiprion* species, including *A. chrysopterus* (Colleye et al., 2011; Colleye & Parmentier, 2012). However, Colleye et al. (2011) averaged, at times, hundreds of sounds from isolated individuals to derive a single metric for each fish, potentially losing any intricacies and complexities of *Amphiprion* vocalisation at a finer (but potentially ecologically valuable) scale. Additionally, previous studies on *Amphiprion* communication have mainly been conducted

under laboratory conditions, with few studies distinguishing between the sexes when assessing vocal communication. Of those which do, the majority described solely male vocal communication (reviewed in Ladich, 2015). This has been attributed to a reproduction-biased research approach, whereby researchers historically focus on males considering they more readily advertise availability and readiness for reproduction through vocal communication than do females (Myrberg et al., 1986; Hawkins, 1993; Hawkins & Amorim, 2000; Ladich, 2015). Of the few studies that have actually assessed male and female sound production, vocalisations were not collected under standardised conditions, nor analysed with rigorous statistical methods, with any descriptions being general and broad (Ladich, 2007; Oliveira, 2014). Therefore, there is a fundamental research gap in: (1) the assessment of individual anemonefish vocalisations in their natural environment, (2) the analysis of variation in the vocal repertoire of individual fish in context and at a fine temporal scale, and (3) the assessment of anemonefish vocalisations dependent on sex, with a predominant gap in the understanding of female vocal communication.

To further our knowledge of *Amphiprion* communication, I studied wild orange-fin anemonefish, *Amphiprion chrysopterus* (Cuvier, 1830), in the lagoonal reef system of Mo'orea, French Polynesia. My aim was to explore how vocalisations and associated behaviours differ in the presence and absence of an "intruder" (in the form of a mirror reflection). Due to the potential for unnatural conditions within a laboratory environment to affect fish behaviour, I recorded vocalisations from *Amphiprion* individuals at natural nest sites, noting other behaviours occurring at the point of vocalisation. Through use of a single-pulse frequency analysis, I aimed to assess anemonefish vocalisations at a finer scale than performed in previous studies (Parmentier et al., 2007; Colley et al., 2009; Colley et al., 2011).

To the best of my knowledge, this is the first study to assess *Amphiprion* vocalisations in the wild, using a single-pulse analysis approach. I tested the hypotheses that:

1. Acoustic signals are more complex and varied *in situ* compared with those reported from laboratory studies.
2. Vocal behaviours is context driven, and more diverse than previously reported from laboratory studies.
3. There are size- and sex- related differences in acoustic communication.

## 2.2 Methods

### 2.2.1 Study Organism

The orange-fin anemonefish (*Amphiprion chrysopterus*) is a site-attached, territorial pomacentrid that has a symbiotic relationship with its sea anemone host *Heteractis magnifica* (Beldade et al., 2016). This site-specific nature makes it a highly tractable species for field studies as sound and video recorders can be positioned in close proximity and left for long periods of time, allowing for natural behaviours to be captured with minimal human disturbance. In addition, orange-fin anemonefish are a social species, where a size-based dominance hierarchy prevails (Fricke, 1979; Buston, 2003a, 2003b; Buston & Cant, 2006). The female is the largest, most dominant fish present in the anemone, with the second largest fish being the most dominant male; this male is submissive to the female but dominant over the other, smaller non-reproductive juveniles (Moyer & Bell, 1976; Hayashi et al., 2020). This allows for relatively easy sex identification in the

dominant breeding pair, enabling sex differences in vocalisations and behaviours to be examined.

### 2.2.2 Study Site

The videos analysed in this study were collected in the lagoonal reef system of Mo'orea, French Polynesia (17°32'11.9" S, 149°49'50.0" W) over the course of two years between 23/06/2015 and 14/06/2017. Behavioural videos were obtained from 60 sites located within the northern reefs of the island.

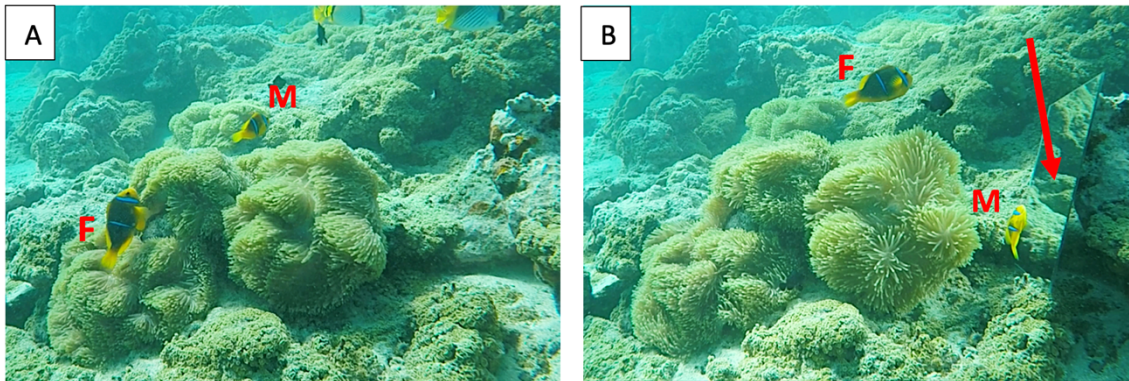
### 2.2.3 Data Capture

#### 2.2.3.1 Method

At each site, a GoPro Hero 4© camera secured with Gorillapods (JOBY©) and dive weights was positioned 2–3 m from a focal anemone hosting a female and male orange-fin anemonefish pair. At more topographically complex sites, two or more cameras were used to capture the behaviour of the pair. Only pairs that were not harbouring and incubating eggs were selected for recording, to ensure that only non-reproductive behaviour was analysed.

After the camera installation, an acclimation period of ca. 5 min was allowed (the maximum time possible to fit the file storage media; Nanninga et al., 2017). This period of acclimation was followed by four *in situ* behavioural trials: (1) baseline trial, (2) novel object challenge, (3) mirror challenge and (4) fish boldness trial, with each trial lasting 4 min. This study focused on a comparison of baseline behaviour vs the mirror challenge (Fig. 2.2), however it must be noted that all fish were exposed to a novel object prior to being exposed to the mirror. For the mirror challenge, a 30 cm x 30 cm mirror was placed within 50 cm of the anemone for 4 min (Fig. 2.2b). Only a few taxa are able to pass the mirror test

(e.g., various primates, De Veer & Van Den Bos, 1999; Schilhab, 2004; grey parrots, *Psittacus erithacus*, Pepperberg et al., 1995; magpies, *Pica pica*, Prior et al., 2008; elephants, *Elephas maximus*, Plotnik et al., 2010; and bluestreak wrasse, *Labroides dimidiatus*, Kohda et al., 2022). Therefore, in these experiments, a mirror should be effective in simulating a ‘rival’ conspecific, likely invoking territorial behaviour in the resident female and male fish.



**Figure 2.2.** Experimental set-up showing a female (F) and male (M) orange-fin anemonefish pair during (A) the baseline period and (B) the mirror challenge, where the mirror (highlighted with a red arrow) is placed within 50 cm of the focal anemone. In image B, the adult M is ‘sizing-up’ his reflection in the mirror (i.e., displaying lateralisation behaviour with the rival conspecific in which he presses his body to the mirror to measure himself against his potential opponent).

Following the behavioural trials, both the adult female and male fish were captured with a hand net by a SCUBA diver, and the total length of each fish was measured with callipers ( $\pm 0.1$  mm). Reef type (lagoon, forereef, pass) at each site was noted, as well as the number of other fish (anemonefish and *Dascyllus trimaculatus*) sharing the host anemone.

#### 2.2.3.2 GoPros

GoPro Hero 4© sport cameras (GoPro™, California, US), were used to record *A. chrysopterus* vocalisation and behaviour. GoPro sport cameras have three internal microphones and a robust underwater housing allowing for collection of

both visual and audio data at depths up to 40 m. Additionally, GoPros record from 0 to 20 kHz, covering the spectral range of most reef organisms, including *A. chrysopterus* (Tricas & Boyle, 2009; Lillis & Mooney, 2016). GoPros are already being used for benthic and fish surveys, as well as for behavioural observations, providing useful insights into underlying fish ecology (Kaplan & Mooney, 2016; Nedelec et al., 2016; Ford et al., 2018; Villon et al., 2018; Lefcheck et al., 2019). GoPros are therefore an appropriate low-cost alternative to more expensive video and sound equipment (Chapuis et al., 2021).

#### 2.2.4 Video Analysis

##### 2.2.4.1 Video Preparation

Prior to analysis, each video was first deemed viable by visually and acoustically scanning the footage. Non-viable videos included those that contained boat noise (which has been previously shown to impact behaviour in this species; Mills et al., 2020), and those in which there were operational problems such as the mirror falling over or the GoPro shifting away from the optimal viewing angle due to strong currents. Additionally, videos where the surrounding soundscape was too loud to obtain clear anemonefish vocalisations were deemed non-viable (e.g., when the host anemone harboured more than 50 *Dascyllus trimaculatus*, or when nearby snapping shrimp dominated the soundscape). In total, 60 videos were recorded from 60 sites, with each single video spanning the four periods (see 2.2.3.1), however, this thesis focuses on the baseline and mirror treatment. Thirty-six of these videos were deemed usable. Of the 24 non-viable videos, five contained boat noise, nine were compromised by operational problems, and 10 had vocalisation masked by the local soundscape.




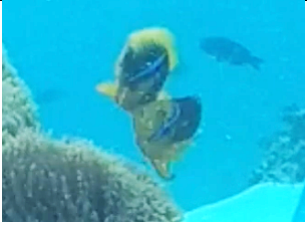
#### 2.2.4.2 Vocalisation Behaviour Analysis

For each fish, two 4 minute periods were analysed, one baseline and one mirror. Each video (n = 36 videos with 1 single video containing both the baseline and mirror period) was analysed twice for both the female and the male fish. Firstly, to identify behaviours associated with vocalisations, I scanned through the videos deemed viable both acoustically (using headphones) and visually (watching the fish on QuickTime Player (Apple Inc)) in order to locate vocalisations. When a vocalisation was found, the behaviour at the time of vocalisation was noted, and from this I formed an ethogram highlighting four vocal behaviours (Table. 2.1). Using this ethogram, I then scored all vocal behaviour occurrences within a 4 minute period during the baseline and mirror periods separately.

The vocalisation behaviours were then transformed into rates (VB min<sup>-1</sup>) per period and compared using a Wilcoxon signed rank test, following normality testing using a Shapiro-Wilk test that found the data did not meet the assumptions required for parametric testing.

It was possible to determine which fish (the male or the female) made each vocalisation, as at the onset of vocalising, *Amphiprion* species elevate their heads and push their lower jaw forwards followed by rapid closure of the lower jaw. Therefore, vocalisations were assigned to specific individuals, including their sex. Where it was not clear which individual was vocalising, these were excluded from analyses comparing between the sexes.

**Table 2.1.** Ethogram of the behaviours that occurred during vocalisations during the baseline and the mirror periods.

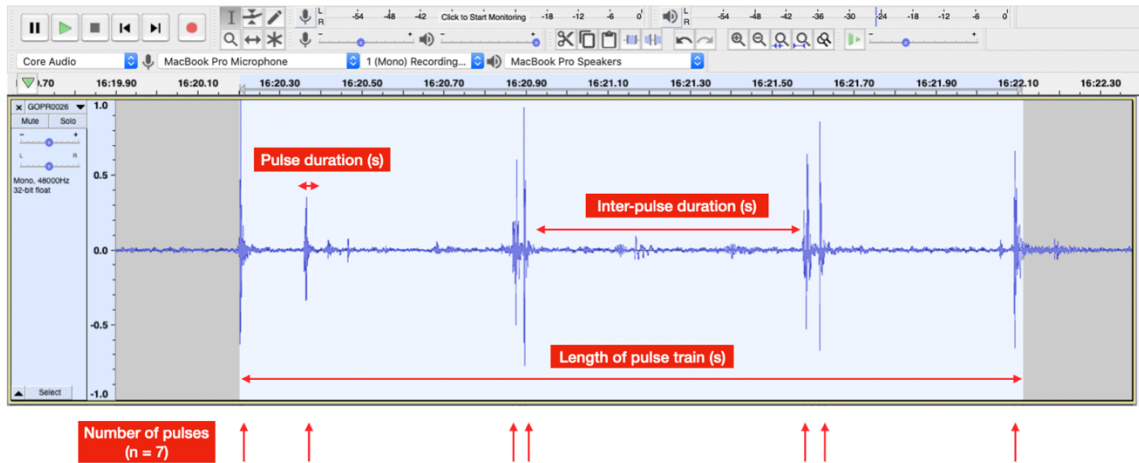
Behaviour Code	Behaviour Type	Description	Image
L	Lateralisation	Anemonefish swims past mirror whilst quivering body against/close to mirror.	
F	Fighting	Anemonefish engages in aggressive confrontation with reflection. This includes aggressively swimming towards and pushing against their reflection in the mirror.	
H	Chasing	Anemonefish rapidly chases another species of fish.	
C	Communication	Female–male anemonefish pair engage in communication with each other. First, they face each other, then they circle each other whilst vocalising.	

### 2.2.5 Sound Analysis

Vocalisations were analysed in Audacity® (Audacity Team, 2021) to obtain key sound metrics: number of pulses within a single train, pulse duration (s), peak frequency of each individual pulse within a train (Hz), inter-pulse duration (s), length of pulse train (s) and peak frequency of the whole vocalisation train (Hz) (Fig. 2.3). Frequencies were calculated by exporting the spectrum with a Hann window with bin widths of 4096 units, as this is the highest resolution possible for such short durations (individual pulses ranged from 7–39 ms). To obtain the frequency of each individual pulse, the single pulse was replicated 10 times to



create a longer sound file. Using this larger file size, the dominant frequency of the single pulse could be calculated at a higher spectral resolution.



**Figure 2.3.** Screenshot of Audacity® (Audacity Team, 2021) showing the sound metrics calculated: number of pulses within a single train, pulse duration (s), inter-pulse duration (s) and length of pulse train (s) (in this example, 7 pulses in a pulse train, highlighted by the vertical seven red arrows at the base of the figure). Frequencies (Hz) were calculated in a separate window using the Audacity Analyze > Plot Spectrum function.

#### 2.2.5.1 Pulse Analysis

After extracting the acoustic metrics of each vocalisation train, the pulses were then analysed for relationships between: pulse peak frequency and sex, pulse peak frequency and fish size, whether different vocalisation behaviours have a characteristic number of pulses, and patterns in pulse peak frequency throughout individual vocalisation trains. A non-parametric Mann-Whitney U test was used to compare the pulse peak frequencies (Hz) obtained for female and male fish.

Linear mixed models (LMMs) were used to assess the relationship between fish size (cm) and pulse peak frequency (Hz), for (1) all fish vocalisations and (2) fish vocalisations separated by sex. LMMs were chosen as I have multiple

pulse peak frequencies from the same individual, which are not therefore independent; LMMs allow the inclusion of a random term for fish identity that takes account of this multiple sampling of the same individual.

To ascertain whether individual vocalisation behaviours had a characteristic number of pulses, bar charts were drawn up and the most frequent number of pulses within a train were studied individually to ascertain which vocalisation behaviours were associated with vocalisations of a particular pulse length. Additionally, peak frequencies of each pulse in vocalisation trains were assessed in rank order to explore patterns within vocalisation trains.

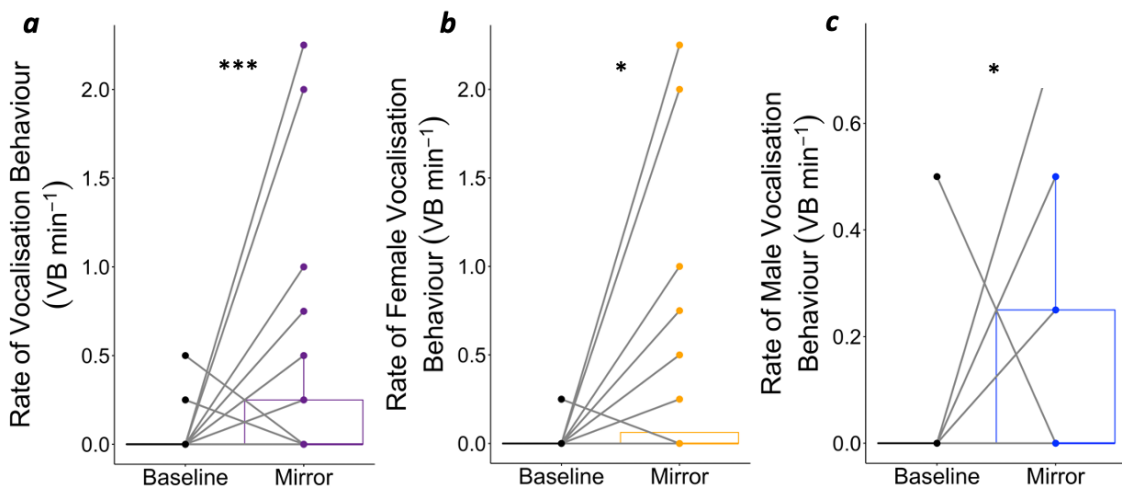
## 2.3 Results

### 2.3.1 Vocalisation Behaviours

Assessment of vocal behaviour in 72 fish (36 male and 36 female fish across 36 sites), 49 fish were non-vocal in both the baseline and the mirror periods. Of the 23 fish that were observed to be vocal, 4 were vocal solely during the baseline period, 19 vocal solely during the mirror period, with none vocal in both periods. A total of 54 vocalisations (37 from 12 females, 17 from 11 males) were found which were then linked to specific behaviours, creating a suite of vocalisation behaviours (Table 2.1).

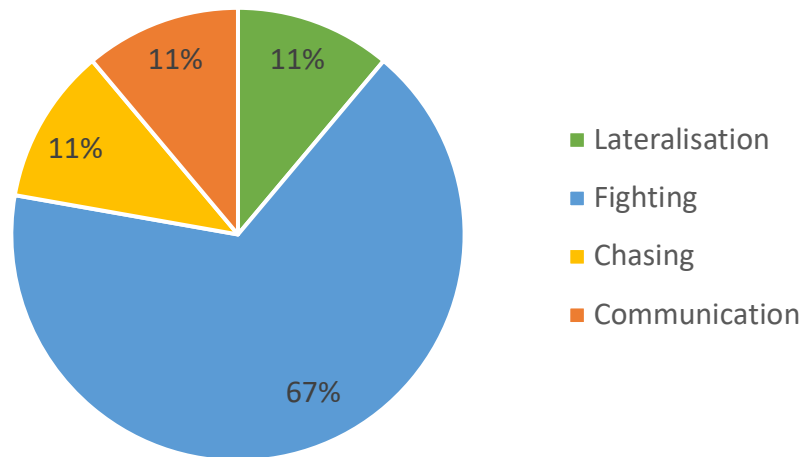
Only five vocalisation behaviours occurred during the baseline period across all the sites, compared to 49 vocalisation behaviours during the mirror period. The rate of vocalisation behaviour ( $\text{VB min}^{-1}$ ) was significantly higher in the mirror period (median = 0.00  $\text{VB min}^{-1}$  (Interquartile range [IQR]: 0, 0.25),  $N = 72$ ), compared to the baseline period (median = 0.00  $\text{VB min}^{-1}$  (IQR: 0, 0),  $N =$

72, Wilcoxon Signed Rank test,  $Z = 245.5$ ,  $p < 0.001$ ; Fig. 2.4a). Additionally, the rate of both female and male vocalisations were significantly higher in the mirror period (female: median =  $0.00 \text{ VB min}^{-1}$  (IQR: 0, 0.06),  $N = 36$  fish; male: median =  $0.00 \text{ VB min}^{-1}$  (IQR: 0, 0.25),  $n = 36$  fish) compared to the baseline period (female: median =  $0.00 \text{ VB min}^{-1}$  (IQR: 0, 0),  $N = 36$  fish;  $Z = 69$ ,  $p = 0.018$ ; Fig. 2.4b; male: median =  $0.00 \text{ VB min}^{-1}$  (IQR: 0, 0),  $N = 36$  fish;  $Z = 58.5$ ,  $p = 0.021$ , Fig. 2.4c).



**Figure 2.4.** Box plots showing the rate of orange-fin anemonefish vocalisation behaviour in both the baseline and mirror period for (a) all 72 fish, (b) 36 female fish, (c) 36 male fish, across 36 sites in Mo'orea, French Polynesia. Grey lines connect data points from the same fish in both periods. \*  $p < 0.05$ ; \*\*\*  $p < 0.001$ .

The most frequent vocalisation behaviour was fighting ( $n = 36$ ), where an individual anemonefish engaged in aggressive behaviours aimed at its reflection (likely perceived as an intruder). The second most common vocalisation behaviours were when a fish 'sized up' its reflection against the mirror (lateralisation;  $N = 6$ ) and engaged in chasing and communication ( $N = 6$ ; Fig. 2.5).



**Figure 2.5.** Vocalisation behaviours displayed by orange-fin anemonefish. Fifty-four instances of vocalisation behaviours were recorded from 23 anemonefish (12 female, 11 male) at 19 sites.

### 2.3.2 Acoustic Metrics of Vocalisations

Vocalisations in the mirror period had a wider range in the number of pulses in a vocalisation train compared to the baseline (Table 2.2). Additionally, a wider range of pulse duration, pulse peak frequency (Hz), pulse peak frequency for whole vocalisation train (Hz), inter-pulse duration (ms) and length of pulse train (ms) was found for vocalisations during the mirror period compared to the baseline period (Table 2.2).

**Table 2.2.** Acoustic metrics calculated from 54 orange-fin anemonefish vocalisations split by period, obtained *in situ* from 19 sites.

Period	No. vocals	No. pulses	Pulse duration (ms)	Pulse peak frequency range (Hz)	Peak frequency range for whole train (Hz)	Inter-pulse duration (ms)	Length of pulse train (ms)
Baseline	5	1–5	12–17 ( $\mu = 13.7$ )	164–574 ( $\mu = 363$ )	199–516 ( $\mu = 379$ )	110–1973 ( $\mu = 525$ )	14–2949 ( $\mu = 1092$ )
Mirror	49	1–9	7–39 ( $\mu = 19.8$ )	117–832 ( $\mu = 443$ )	11–832 ( $\mu = 379$ )	8–4530 ( $\mu = 583$ )	7–12082 ( $\mu = 1286$ )
<b>Total</b>	<b>54</b>	<b>1–9</b>	<b>7–39</b> <b>(<math>\mu = 19</math>)</b>	<b>117–832</b> <b>(<math>\mu = 439</math>)</b>	<b>11–832</b> <b>(<math>\mu = 379</math>)</b>	<b>8–4530</b> <b>(<math>\mu = 580</math>)</b>	<b>7–12082</b> <b>(<math>\mu = 1276</math>)</b>

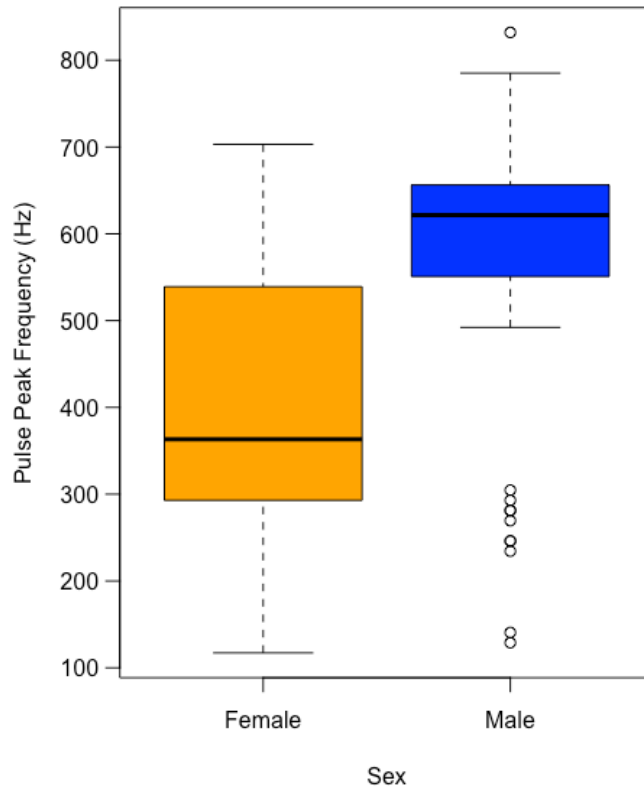
Vocalisations in each behavioural category had a wide range of number of pulses, but communication behaviours included the greatest number of pulses in a train (Table 2.3). Mean pulse duration and pulse peak frequency (for both individual pulses and the whole train) were similar across all behavioural groups (Table 2.3). Lateralisation vocalisations consisted of pulses that were closer together within trains (smaller inter-pulse duration), and shorter trains overall (shorter length of pulse train) compared with the other three behaviours (Table 2.3).

**Table 2.3.** Acoustic metrics calculated from 54 orange-fin anemonefish vocalisations split by behavioural group, as obtained from 19 sites.

Behaviour	No. vocals	No. pulses	Pulse duration (ms)	Pulse peak frequency range (Hz)	Peak frequency range for whole train (Hz)	Inter-pulse duration (ms)	Length of pulse train (ms)
L	6	1–6	13–23 ( $\mu = 18.6$ )	270–785 ( $\mu = 406$ )	35–352 ( $\mu = 272$ )	115–684 ( $\mu = 209$ )	14–969 ( $\mu = 563$ )
F	36	1–6	7–39 ( $\mu = 20.0$ )	117–832 ( $\mu = 446$ )	35–832 ( $\mu = 402$ )	8–4211 ( $\mu = 909$ )	7–12082 ( $\mu = 1452$ )
H	6	1–6	12–23 ( $\mu = 15.5$ )	164–738 ( $\mu = 496$ )	12–703 ( $\mu = 391$ )	109–1973 ( $\mu = 360$ )	14–2947 ( $\mu = 890$ )
C	6	2–9	14–35 ( $\mu = 22.0$ )	176–644 ( $\mu = 404$ )	35–597 ( $\mu = 333$ )	25–1426 ( $\mu = 330$ )	370–3651 ( $\mu = 1314$ )

#### 2.3.2.1 Pulse Peak Frequency ~ Fish Sex

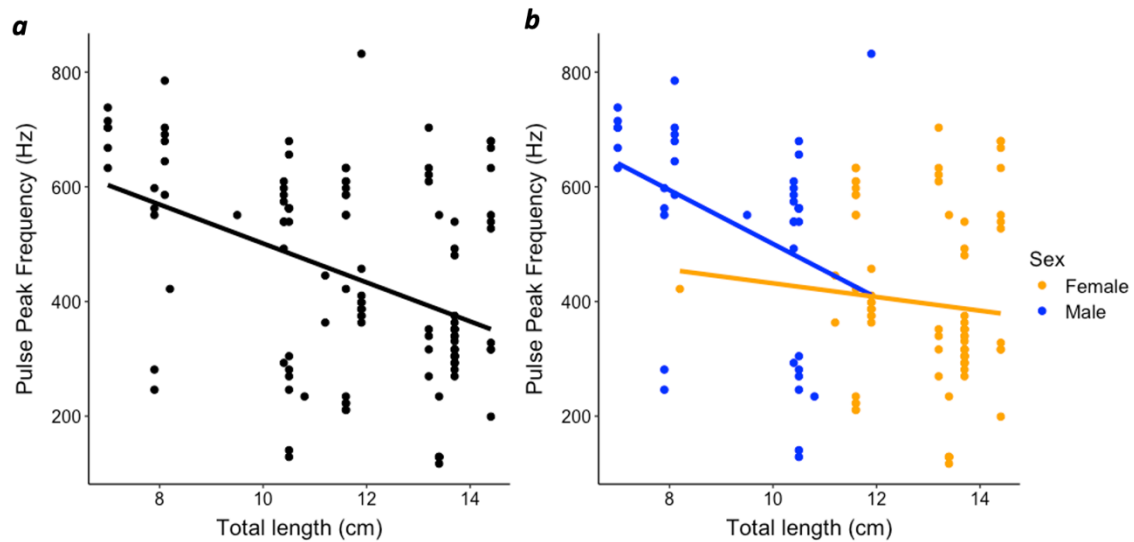
The mean peak frequency of pulses made by female fish was approximately 1.11 standard deviations lower (N = 89 pulses from 10 females, mean = 397 Hz, median = 363 Hz (IQR: 305, 539) than males (N = 63 pulses from nine males, mean = 572, median = 622 Hz (IQR: 492, 668)), (Wilcoxon Signed Rank test:  $U = 1228$ ,  $p < 0.001$ ; Fig. 2.6).



**Figure 2.6.** Box plot showing the pulse peak frequency (Hz) of female (orange;  $n = 89$  pulses) and male (blue;  $N = 63$  pulses) orange-fin anemonefish. Outliers depicted by circles; \*\*\*  $p < 0.001$ .

### 2.3.2.2 Pulse Peak Frequency ~ Fish Size

A negative relationship between body size and pulse peak frequency was found, but this relationship was not statistically significant (LMM:  $t = -1.878$ ,  $p = 0.083$ ; fish ID [random term]: variance  $\pm$  SD =  $13439 \pm 115.9$ ; Fig 2.7a). There was no evidence of a size~sex interaction (LMM:  $t = -0.648$ ,  $p = 0.526$ ; fish ID [random term]: variance  $\pm$  SD =  $14211 \pm 119.2$ ; Fig 2.7b).

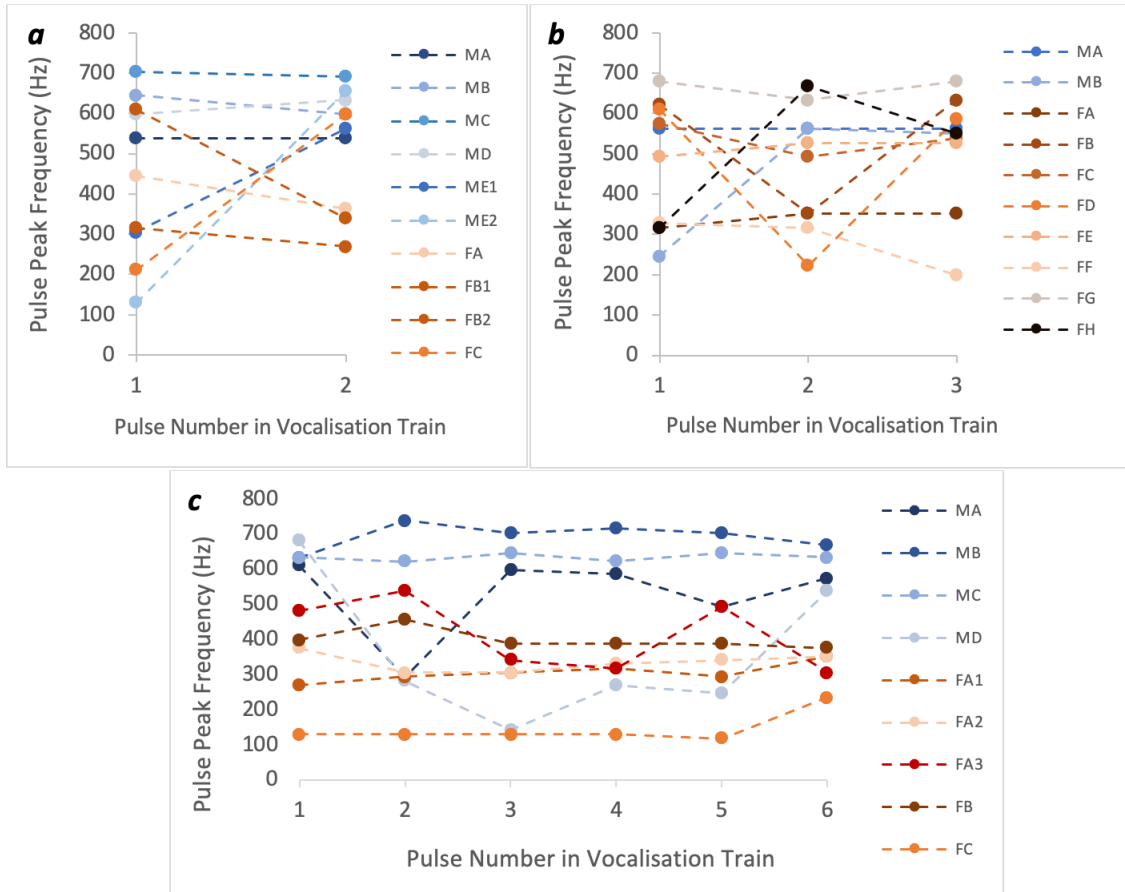


**Figure 2.7.** Influence of fish total length (cm) on non-averaged pulse peak frequency (Hz) in orange-fin anemonefish across (a) all 19 vocalising fish (118 pulses) and (b) split by sex (10 females in orange = 78 pulses; nine males in blue = 40 pulses). Each data point represents a single pulse, with a vertical line of data points representing a single fish.

### 2.3.2.3 Patterns of Pulse Peak Frequency Within a Vocalisation Train

Assessment of patterns within vocalisation trains highlighted that pulse frequencies can vary substantially between pulses (Fig. 2.8). Two-pulse trains could increase, decrease or remain similar in frequency between the pulses (Fig. 8a), and three-pulse trains showed even greater complexity (Fig. 2.8b). Within a single six-pulse vocalisation train, one male (Fig. 2.8c, individual MD), produced a series of pulses, with peak frequencies of 680 Hz, 281 Hz, 141 Hz, then climbing back up to finish with the sixth pulse at 539 Hz. This range of pulse peak frequencies within a vocalisation train was also seen within female vocalisations. For example, a female (Fig. 2.8b, individual FD) initiated a three-pulse vocalisation train with a pulse at 610 Hz, then dropped to 223 Hz, before finishing back at 586 Hz for the final pulse.





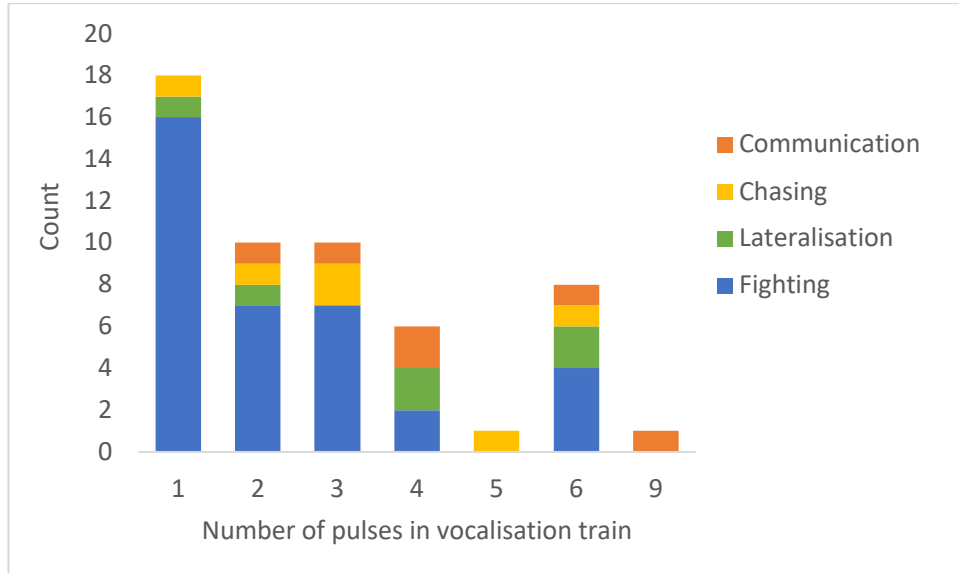
**Figure 2.8.** Patterns of individual pulse peak frequencies (Hz) within orange-fin anemonefish vocalisation trains of (a) two-pulses, (b) three-pulses and (c) six-pulses. Blue lines = male vocalisation trains; orange lines = female vocalisation trains. Legend denoting the sex (M or F) and the individual (A, B, C, D), when an individual fish has more than one vocalisation train (1, 2, 3 etc.) is added to the end (i.e. FA1, FA2, FA3).

Many individuals also vocalised at around the same peak frequency for each pulse within a vocalisation train (Fig. 2.8c). For example, male c (MC) vocalised consistently between 621 Hz and 645 Hz throughout a six-pulse train.

#### 2.3.2.4 Number of Pulses

Shorter vocalisation trains were more common than longer trains. One third of vocalisations were one-pulse long, two and three-pulsed trains occurred in 19%

of cases, and vocalisation trains comprising more than three pulses generally contained six pulses (15% of vocalisations, Fig. 2.9).



**Figure 2.9.** Count of pulse trains with different numbers of pulses obtained from 54 vocalisation trains from 23 orange-fin anemonefish (12 female, 11 male) at 19 sites across the northern shore of Mo’orea, French Polynesia. Individual bars are colour coded relative to the behaviours displayed at time of vocalisation.

Pulse length did not appear to be related to behaviour, with 1, 2, 3, 4 and 6 pulsed vocalisation trains used when the anemonefish were fighting, and 2, 3, 4, 6 and 9 pulsed vocalisations used when communicating (Fig. 9).

## 2.4 Discussion

The overarching aim of this study was to take a deeper dive into anemonefish communication recorded *in situ*, analysing natural vocalisations obtained from wild orange-fin anemonefish in the reefs of Mo’orea, French Polynesia. The main finding from this individual pulse-based analysis is that vocal repertoires and vocalisation behaviour in anemonefish are complex and diverse. Introducing a

'rival' conspecific anemonefish (by means of a mirror reflection) into the natural environment allowed agonistic anemonefish vocalisations and behaviours to be assessed, resulting in the production of the first ethogram depicting wild vocalisation behaviours in anemonefish. The data presented here suggest that orange-fin anemonefish does not commonly engage in acoustic communication, but when a conspecific intruder enters the vicinity of the anemone, vocalisations and aggressive behaviours increase significantly, with anemonefish vocalising most frequently when engaged in fighting with a perceived 'rival' anemonefish. Acoustic metric analysis highlighted that these vocalisations were not related to specific behaviours. Additionally, the data here support the theory that anemonefish pulse peak frequency differs between female and male fish, supporting the initial hypotheses. However, in opposition to the original hypothesis, pulse peak frequency was not found to differ dependent on fish size. For the first time, this study shows that both male and female orange-fin anemonefish can vocalise at varying frequencies within a vocalisation train.

#### 2.4.1 Vocalisation Behaviours

Vocalising animals have to trade-off the need to communicate and the risk of being overheard by a predator or competitor (Deecke et al., 2005; Lobel et al., 2010; Lourenço et al., 2013; Maiditsch & Ladich, 2022). The low vocalisation behaviour rate found within the baseline period suggests that orange-fin anemonefish produce sound infrequently, which, from an ecological and evolutionary perspective, would be beneficial for the avoidance of nearby predators. The 10-fold increase in vocalisations found during the mirror period could suggest that the perceived threat of a rival anemonefish is sufficient to outweigh threats resulting from nearby predators or competitors intercepting the sound. In *Amphiprion* species, the position of the resident fish in the group hierarchy is primarily dependent on body size (Fricke, 1979; Buston, 2003a; Buston & Cant, 2006). Non-breeders form a queue for breeding position (Buston, 2004b), therefore, if a potential intruding anemonefish is of a similar size to that

of the resident fish, then the incorporation of the intruding individual into the group could threaten the resident fish's position in the dominance hierarchy. Considering the most dominant male mates with the dominant female, being relegated in the hierarchy carries a potentially severe fitness cost in terms of reduced likely matings for the male fish (Buston, 2004b). Conversely, for females, losing a fight with a larger intruding female could result in death. Therefore, in this example, it is possible that the need to communicate to deter a conspecific intruder that is likely to challenge the resident fish's social position outweighs the risk of being detected by a predator.

Nineteen of the studied female–male pairs did not vocalise during the mirror period, despite the addition of the perceived intruder. This could be explained by the aforementioned trade-off; the fish here avoided vocal behaviour because of the threat of a nearby predator. Furthermore, 17 of these female–male pairs were non-vocal during the baseline period. This may be due to the anemonefish's 'personality type' having an influence on individual fish behaviour. Personality has been shown to influence behaviour in mammals (Wilson et al., 1994; Svartberg et al., 2005), reptiles (López et al., 2005) and other fishes (Sneddon, 2003; Bell, 2005; Yoshida et al., 2005). Inter-individual differences, frequently characterised as the degree of boldness, can profoundly affect behaviour of animals in specific situations and environments (Frost et al., 2007). Therefore, fish personality type (i.e., bold or shy) could influence anemonefish vocalisation. Wong et al. (2013) highlighted consistent individual differences in activity, boldness and sociability for *Amphiprion ocellaris* and boldness and aggression for *A. mccullochi* (Wong et al., 2017), but conversely found inconsistent differences across boldness, aggression and sociability for *A. latezonatus* (Wong et al., 2017). This behavioural inconsistency in closely related species may be due to differences in environmental and social contexts (Wong et al., 2017). Therefore, further work is required to ascertain whether the individual differences in the behaviours seen in this present study on *A. chrysopterus* are consistent throughout time and between different environmental and social contexts, and thus influenced by personality type. Further study could

also test for interactions between potential nearby threats and personality type in determining likelihood of exhibited vocal behaviour.

As predicted, a range of vocalisation behaviours were observed in this present study. However, the majority of behaviours were aggressive in nature, occurring predominantly in the mirror period, with the lower level of aggressive vocal behaviours seen in the baseline period being characteristic of a stable social group, in which conflict is rarely expressed (Wong et al., 2016). Previous studies have used mirrors to invoke aggression in a range of species, particularly in fish aggression studies conducted in the laboratory (Earley et al., 2000; Verbeek et al., 2007; Wilson et al., 2011; Balzarini et al., 2014). The present study found a significant increase in the rate of vocalisation behaviour in the mirror period, highlighting the mirror as an effective tool for invoking aggression and vocalisation behaviour in the wild. Mirrors are beneficial as they require fewer fish, avoid pseudoreplication on captured 'intruders', and prevent welfare issues that arise when two highly aggressive fish are allowed to make contact (Elwood, 1991). Additionally, the effect of fish size on the opponent's behaviour is controlled for because, in each replicate, the experimental subject is confronted with a conspecific 'intruder' of exactly the same size. This optimises quantification of aggressive behaviours (Tinbergen, 1951; Gallup, 1969;). However, the mirror trial used here is limited because the only information provided to the focal fish is visual. Therefore, there is potential that the resulting focal fish behaviour seen here is different from that in situations where more information, including olfactory and acoustic cues, is available (Balzarini et al., 2014). Previous studies have already highlighted this downfall, with mirrors failing to elicit expected hormonal responses (Oliveira et al., 2005; but see Dijkstra et al., 2012) and predicted gene expression in the brain (Desjardins & Fernald, 2010), when compared to contests with live adversaries. The benefit of using real intruders is that they perform natural behaviours that initiate natural responses in the resident focal fish, whereas a mirror reflection simply displays the behaviour performed by the focal fish, with the mirror failing to fully replicate reality. For example, at the beginning of a contest, fish commonly display their flanks (lateralisation – as seen in this

study) to ascertain the physical attributes and the potential threat of the conspecific intruder (Enquist et al., 1990; Hurd, 1997; Arnott & Elwood, 2009a, 2009b). Fish align by either facing the same direction as each other (head-to-head) or by facing in opposite directions (head-to-tail). Considering the mirror used here shows a complete reflection of the focal fish, head-to-tail behaviours do not occur. Therefore, caution should be exercised when interpreting behavioural results from the mirror presentation performed here and therefore, for rigorous behavioural analysis to be conducted in wild orange-fin anemonefish, both a combination of mirror and live opponent trials are necessary to obtain complete behavioural responses (Balzarini et al., 2014). Future studies using mirrors should also aim to incorporate other cues, such as acoustic cues; as in Stout (1975, 1983), where satinfin shiners (*Notropis analostanus*) were exposed to both mirrors (visual cue) and vocalisation playbacks (acoustic cue).

Despite limitations of mirrors, they provide a valuable tool for invoking aggressive behaviours in fish, as demonstrated by this study. Earley et al. (2000) found that mirrors elicited the strongest aggressive response in mangrove killifish (*Rivulus marmoratus*), followed by a dummy fish and then a standard opponent. Likewise, Balzarini et al. (2014) found that daffodil cichlids (*Neolamprologus pulcher*) showed a significant lack of constraint in aggressive behaviours towards their mirror image compared to that of a live opponent. In light of this, and the significantly increased rate of aggressive behaviours and vocalisations in the mirror period cf. the baseline period observed in this study, mirrors clearly provide a valuable tool to invoke strong aggressive responses allowing for the collection of *in situ* vocalisations in wild populations of anemonefish.

#### 2.4.2 Acoustic Metrics of Vocalisations

Fish species in the family Pomacentridae are some of the most extensively studied species in terms of acoustic communication (Schneider 1964). All 27

*Amphiprion* species produce sounds, allowing for comparisons to be drawn between species of the same genus (Colleye et al., 2011). In this present study, orange-fin anemonefish (*A. chrysopterus*) were found to have vocalisations ranging from 1 to 9 pulses, similar to that found in *Amphiprion clarkii* (1–8 pulses; Parmentier et al., 2007). Previous studies have postulated that differences in the number of pulses between *Amphiprion* species may be due to the behaviour and motivational state of the individual fish (Myrberg et al., 1965; Parmentier et al., 2010). However, the results suggest otherwise in that no link was found between behaviour and number of pulses across all four behavioural groups (e.g., fighting vocalisations were 1, 2, 3, 4 and 6 pulses long). Mean inter-pulse duration was approximately 1.2 seconds longer for *A. chrysopterus* than *A. clarkii*, but mean pulse duration (*A. chrysopterus*: 19 ms; *A. clarkii*: 23 ms) and pulse peak frequency range (*A. chrysopterus*: 117–832 Hz; *A. clarkii*, 450–800 Hz) was similar for the two species. Considering the same sonic ligament (*c-md* ligament) is present in other members of the damselfish family, the similarities across these two species are unsurprising, especially as they are similar in size (maximum size: *A. chrysopterus*, 17 cm; *A. clarkii*, 15 cm). The mean pulse peak frequency found here (411 Hz) was very similar to that obtained in a laboratory study assessing vocalisations for *A. chrysopterus* (438.64 Hz; Colleye et al., 2011). Additionally, average pulse duration in this study (19 ms) is also similar to that reported previously for *A. chrysopterus* (18.9 ms; Colleye et al., 2011).

This study, by measuring the peak frequencies of each pulse within vocalisation trains, highlighted, for the first time, the range of frequencies present within single vocalisation trains in orange-fin anemonefish. Past studies have shown that some fishes vocalise consistently within a specific range of frequencies (Lindström & Lugli, 2000; Amorim et al., 2013). However, this study found otherwise, with vocalisation trains including substantial variations in pulse peak frequency, for example from 680 to 281 Hz in less than 0.66 seconds. The mechanism behind this significant jump in frequency is unknown. Potentially, anemonefish have greater control over the frequencies at which they vocalise than previously observed. Parmentier et al. (2016) found that fish can adopt

temporal and frequency partitioning to overcome acoustic masking, increasing the likelihood of the full range of information of the signal reaching the intended receiver. However, no studies have found frequency modulation within single vocalisation trains before, with Ladich (2015) stating ‘there is no report to date on the ability to change sound amplitude in fish’. Insects, anurans and birds, have been proven to regulate sound according to specific social conditions and motivational contexts (Wyman et al., 2008; Nemeth et al., 2012), and potentially anemonefish should be added to this list. Further work is needed to identify the mechanism underpinning the results found here.

This is the first study to distinguish, analyse and compare the sound characteristics of natural vocalisations obtained *in situ* for both sexes of a fish species under standardised conditions. Past studies on fish vocalisations have focused predominantly on males, with a particular emphasis on reproductive behaviour, given that males vocalise more frequently to advertise their condition and nest sites (Myrberg et al., 1986; Hawkins, 1993; Hawkins & Amorim, 2000; Bass & McKibben, 2003). To date, the few studies comparing male and female sound production have described vocalisations generally and without statistical analyses (Lagardère et al., 2005; Ladich, 2007; Oliveira et al., 2014), with formal investigation into the characteristics of female vocalisations lacking. In hermaphroditic anemonefish, females are the most aggressive sex and so provide an ideal system to bridge this gap. In support of the original hypothesis in this study, significant differences in sound characteristics (more specifically pulse peak frequency) between female and male orange-fin anemonefish were found. This contrasts with previous studies where minimal differences in sound characteristics were found between the sexes for different species to the study species assessed here (e.g., three cichlid species, Myrberg et al., 1965; *Cottus gobio*, Ladich, 1990; two *Carapus* species, Lagardère et al., 2005), most likely due to limited sexual dimorphism in the study species. The significant sex differences in vocalisations in this study are most likely due to differences in body size seen between males and females, which affects frequencies of sound produced by acoustic apparatus scaled to body size. Larger female orange-fin



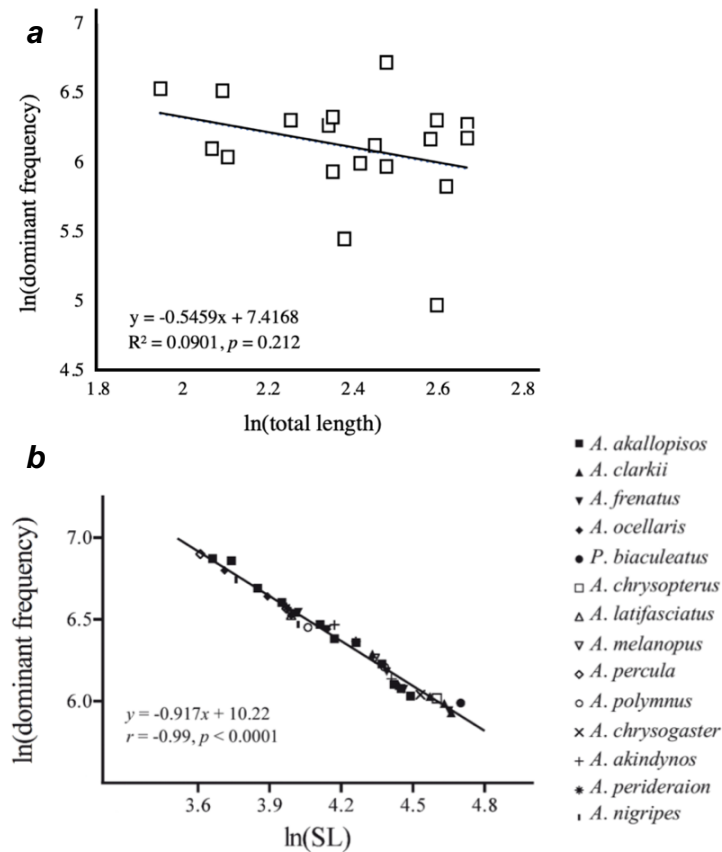
anemonefish possess larger sonic muscles (muscles which produce twitches of a greater duration and thus a lower frequency (Connaughton et al., 2000), and generally vocalised at lower frequencies. However, caution should be taken when assigning fish sex based solely on dominant frequency, as the pulse peak frequency of individual pulses within pulse trains overlaps between the sexes.

Individual sounds produced by an individual can convey information in addition to the main motivated signal, for example about body condition, health and aggressivity (Parsons et al., 2017). Schuster (1986) found that in dwarf gourami (*Colisa lalia*), aggressive displays coupled with vocalisations were more effective than aggressive attacks without vocalisations, with 31% of intruders fleeing quickly over longer distances when experiencing vocalised aggression, compared to 5% in non-vocal attacks. Additionally, Ladich (1990) found that vocalisation was more important in predicting success in a fight than body size. This may explain why orange-fin anemonefish individuals vocalised primarily when fighting (67%) in this study. Vocalisations could help to advertise individual levels of aggression (i.e., willingness to attack and defend a territory). These studies highlight the importance of vocalisation during aggressive attacks to aid in deterring intruders, helping to explain the high proportion of vocalisations associated with fighting in this study.

In opposition to my original hypothesis that there are size-related differences in acoustic communication, this present study found a non-significant, negative correlation between fish size and pulse peak frequency. Nonetheless, a trend was found relating to size and frequency (Fig. 2.7a). Significant trends in averaged pulse peak frequencies and size have been reported for myriad other fish species (e.g., cichlid species (*Hemichromis bimaculatus*, *Cichlasoma nigrofasciatum*, *Pterophyllum* sp.), Myrberg et al., 1965; *Trichopsis vittata*, Ladich et al., 1992; *Pomacentrus partitus*, Myrberg & Shablott, 1993; *Dascyllus albisella*, Lobel & Mann, 1995; *Pollimyrus adspersus* and *P. isidori*, Crawford et al., 1997; *Cynoscion regalis*, Connaughton et al., 2000; *Oreochromis*

*mossambicus*, Amorim et al., 2003; *Eutrigla gurnardus*, Amorim & Hawkins, 2005; *Parablennius parvicornis*, De Jong et al., 2007; *Amphiprion akallopisos*, Colleye et al., 2009; *Amphiprion frenatus*, *A. ocellaris* and *A. clarkii*, Parmentier et al., 2009; *Astatotilapia burtoni*, Maruska et al., 2012; *Pomatoschistus pictus*, Amorim et al., 2013). Previous studies have suggested that size-based variation in frequency is most probably due to the dimensions of the individual's vocal apparatus, in particular the swimbladder – a key structure that aids in sound resonance (the larger the swimbladder, the lower the frequency resonated; Ladich, 2015). This is corroborated by studies on species without swimbladders (e.g., toadfishes, batrachoidids; Bass & McKibben, 2003) that found no correlation between fish size and peak frequency. Therefore, the non-significant result found in this present study may be (1) due to the increased variation found when using single pulse frequencies from individual fish in the analysis, or (2) a result of an insufficiently large sample size, considering a trend between size and frequency was found. Furthermore, I found no significant sex difference in relation to pulse peak frequency, either as a main effect or when interacting with size. Figure 2.7b suggests that there might perhaps be a sex difference in this relationship but further work is needed to explore non-averaged pulse peak frequency ~ size relationships with larger sample sizes whilst additionally assessing for differences relating to sex.

In an earlier study on orange-fin anemonefish. Colleye et al. (2011) reported a significant correlation between fish size and dominant frequency (averaging across pulses within trains to derive a single frequency for each individual). To compare this study with Colleye et al. (2011), pulse trains were averaged and ln(transformed) to give comparative metrics (Fig. 2.10).



**Figure 2.10.** The influence of total length on average pulse frequency (Hz) in (a) 19 orange-fin anemonefish individuals in this study, and (b) across 14 species of anemonefish (Colley et al., 2011). Results are expressed as averages of pulses for each individual fish (number of pulses that were averaged ranged from: (a) 1–25 pulses and (b) 50 pulses).

Although I found a similar negative pattern when averaging pulse peak frequencies, in this case, a non-significant trend was observed, highlighting the effect of additional variation captured by analysing single pulses within trains. This difference may also relate to the different methodologies used. The present study collected all sounds *in situ* whereas Colley et al. (2011) obtained some sounds from the lagoon and others from tanks, although it is unclear on the numbers of this split. Potentially, fish vocalising *in situ* were under a wider range of environmental and social contexts than present in the laboratory, resulting in a greater variation in vocalisations obtained here. In earlier work, where the calling individual can be identified, multiple sounds from individuals have been averaged into a single metric (Fine & Parmentier, 2015). This may be useful in laboratory-

based experiments, where the manipulation and handling of many vocalisations by each individual is made simpler through averaging. However, when vocalisations can be captured *in situ* and attributed to an individual, there is increased value in examining single pulses within each vocalisation train.

Adopting a single-pulse analysis approach, this study identified previously undocumented variation in orange-fin anemonefish vocalisation and associated behaviour, suggesting that by averaging, valuable information about ecologically relevant complexity in vocalisation behaviour has, until now, gone undiscovered. In conducting these analyses, I have highlighted anemonefish as being a highly tractable and useful model species for fish vocalisation research in the wild.

## Chapter 3:

*In situ* assessment of effects of artificial light at night (ALAN) on the orange-fin anemonefish *Amphiprion chrysopterus*

### 3.1 Introduction

Light has been a fundamental and reliable environmental cue throughout history, facilitating the orchestration and timing of biological activity for many organisms. Repeating natural fluctuations in light from celestial bodies, such as the sun, moon and stars, have led to highly preserved biological clocks, controlling circadian and circannual rhythms that are vital in the regulation and coordination of biological behaviours and physiology (Longcore, 2010; Gaston et al., 2013; Brüning et al., 2018). With the widespread discovery of gas and electricity dating back to the late 1800s (Longcore et al., 2010), human activities have, and continue to, generate significant levels of artificial light. Now classified as a major pollutant, artificial light at night (ALAN) originates from human infrastructure, such as streetlights found in towns, cities, harbours and docks (Gaston et al., 2015) and is pervasive in both terrestrial and aquatic ecosystems. This artificial light shines upwards and reflects off clouds, producing a secondary, indirect source of ALAN named 'skyglow' (Kyba et al., 2011). As the human population grows, increased urbanisation and associated light-emitting infrastructure are predicted to brighten the Earth by ~2.2% each year (Kyba et al., 2017). With light pollution ever-increasing, unpredictable light and dark cycles can influence on highly evolved circadian and circannual rhythms of myriad organisms. This can cause significant impacts at species, population and ecosystem levels worldwide (Gaston et al., 2015; Davies & Smyth, 2017; Sanders et al., 2021).

Early studies on the ecological impacts of ALAN primarily focused on the more obvious impacts of light pollution, predominantly in terrestrial species, e.g., the effect of street lights in attracting insects and birds or delaying leaf-fall on trees (Matzke, 1936; Howell, 1954; Verheijen, 1960). More recent research has explored the implications of ALAN on a extensive array of biological phenomena across a wide range of taxa, from mammals and reptiles to amphibians and invertebrates (Stone et al., 2009; Spoelstra et al., 2015; Sanders et al., 2021). Marked changes in physiological processes and behavioural actions occur as a

result of exposure to ALAN, with confirmed effects on: migration (Stone et al., 2009; Van Grunsven et al., 2017), activity and metabolism (Rotics et al., 2011; Pulgar et al., 2019), immune function (Becker et al., 2020; Durrant, et al., 2020), growth and survival (McLay et al., 2017; Schligler et al., 2021), reproduction (Fobert et al., 2021; Sanders et al., 2021) and community structure (Manríquez *et al.* 2019). In contrast to this large body of research in terrestrial systems, far fewer studies have examined effects of ALAN in aquatic environments with only 20% of papers studying effects of light pollution focus on aquatic species. This is surprising considering 25% of the human population live along the world's coastlines (Small & Nicholls, 2003), with considerable associated light-production from e.g., oil platforms, hotels, fisheries, streetlights, boardwalks, piers and ships. This light pervades underwater and presents a real threat to marine species living within these coastal areas (Davies, et al., 2014, 2016). Nonetheless, very few of the limited number of fish studies consider species inhabiting coastal regions (Foster et al., 2016; Bolton et al., 2017; Schligler et al., 2021). Additionally, only 8% of taxa-specific publications on the effect of ALAN focus on fishes (Bassi et al., 2021). This seems disproportionate considering fishes represent the largest and most diverse group of vertebrates, constituting >35,000 species (Ravi & Venkatesh, 2008; WWF, 2021). Therefore, further research is undeniably needed to examine potential negative ecological impacts of ALAN on marine fishes and ecosystems (Gaston & Bennie, 2014; Davies et al., 2015).

To date, ALAN studies on adult fish behaviour have generally focused on reproductive behaviour. For example, parental-care activity significantly increased in nest-guarding male smallmouth bass (*Micropterus dolomieu*) during exposure to shoreline light in freshwater ecosystems, potentially depleting energy reserves (Foster et al., 2016). This research focus on reproductive behaviour is driven by the need to assess impacts on the replenishment of future populations (Fobert et al., 2019, 2021; O'Connor et al., 2019). However, it is also important to study non-reproductive behaviours. Fish display a wide range of non-reproductive behaviours, such as social interactions (e.g., aggressivity, sociability) and feeding behaviours; behaviours fundamental

to individual health and subsequent fecundity. These behaviours have already found to be significantly influenced by artificial light (Réale & Dingemanse, 2010). Two studies conducted at night found that the intertidal rockfish (*Girella laevis*) displayed heightened activity levels with elevated metabolic rates during exposure to ALAN (Pulgar et al., 2019), and Trinidadian guppies (*Poecilia reticulata*) showed greater boldness when exposed to ALAN in the laboratory, with fish spending significantly more time within the open areas of the behavioural tanks and away from the walls, potentially exposing them to greater levels of predation (Kurvers et al., 2018). This previous work demonstrates that ALAN has the potential to influence non-reproductive nocturnal fish behaviour, but much less is known about how diurnal behaviour is influenced following night-time exposure to ALAN.

Coral reefs are major hubs of biodiversity, harbouring approximately 25% of known marine species (Plaisance et al., 2011). The site-attached nature of many species on reefs, including the damselfish (Pomacentridae) that can make up 50% of total fish biomass in coral reef fish communities (Ackerman & Bellwood, 2000), means that many reef fish have intensified social interactions with neighbouring hetero- and conspecifics (Helfman & Schultz, 1984; Wong et al., 2007). However, being site-attached limits their ability to migrate to more favourable environments following exposure to local stressors. In spite of the disproportionately high exposure to ALAN in coastal regions (Grimm et al., 2008), the indirect effect of ALAN on diurnal behaviour in coral reef fishes, following exposure at night, has not been extensively studied (but see: Fobert et al., 2021; Schligler et al., 2021).

To examine the effect of ALAN on a coral reef fish, I tested whether exposure to ALAN impacts subsequent diurnal behaviour of orange-fin anemonefish (*Amphiprion chrysopterus*). Orange-fin anemonefish live on coral reefs, in symbiotic relationship with their host anemone the magnificent sea anemone (*Heteractis magnifica*) (Fig. 3.1). They live in groups with a social size-



based dominance hierarchy, involving highly evolved social behaviours (Moyer & Bell, 1976; Hayashi et al., 2020). These groups are site-attached to reef habitat, located close to shore, and often in close proximity to humans and associated anthropogenic stressors, including light and sound pollution. Using female–male pairs in a field-based experiment, I explored how exposure to ALAN influenced four non-reproductive diurnal behaviours (Social, Aggression, Vocalisation,



**Figure 3.1** Orange-fin anemonefish individual nestled within the tentacles of its host, the magnificent sea anemone (*Heteractis magnifica*). Anemonefish frequently share the host anemone with other anemonefish, such as *Dascyllus trimaculatus* (see top left of image, black caudal and pectoral fin) (image: Frederick Zuberer).

Hiding) following exposure to either natural light at night (control treatment; ~0.03 lux) or exposure to artificial light at night (ALAN treatment; ~12–26 lux) at the home anemone. Diurnal behaviour was sampled at three periods: BEFORE treatment commenced, after IMMEDIATE exposure to treatment (one night) and after PROLONGED exposure to treatment (~14–35 nights).

In this study, I predicted that light pollution at anemonefish colonies would have a significant impact on subsequent diurnal behaviour. I predicted that there would either be:

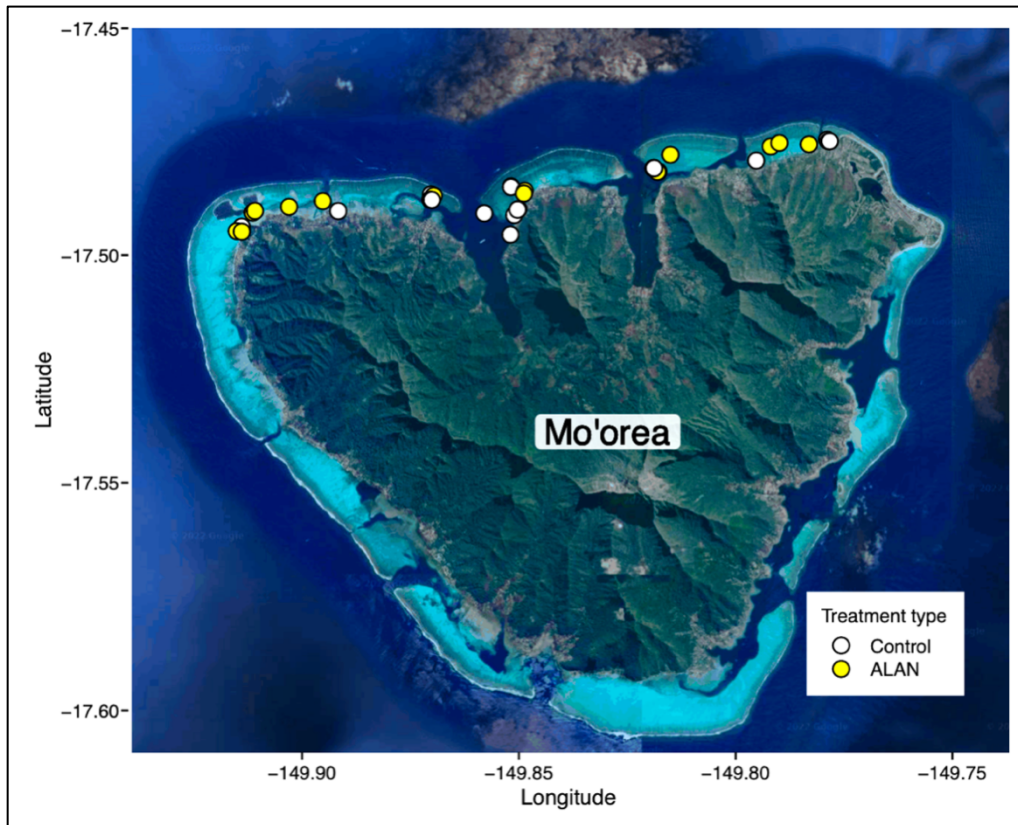
1. a minimal negative effect after IMMEDIATE exposure to ALAN, but that this would intensify with PROLONGED exposure, due to sensitisation; or
2. a large, negative effect after IMMEDIATE exposure to ALAN, but that this would lessen with PROLONGED exposure, due to acclimation (as seen with noise pollution in this species; Mills et al., 2020); or

3. a large, negative effect after IMMEDIATE exposure that is maintained even after PROLONGED exposure.

## 3.2 Methods

### 3.2.1 Study Site

The non-reproductive behavioural response of the male-female orange-fin anemonefish pairs to both IMMEDIATE (one night) and PROLONGED (range: 14–35 nights, mean:  $20.67 \pm 0.94$  SE) experimental exposure to ALAN was tested along the northern shore of Moorea, French Polynesia, Pacific Ocean ( $17.5^{\circ}\text{S}$ ,  $149.8^{\circ}\text{W}$ ; Fig. 3.2) during January to April 2021. To standardise behavioural state, anemonefish reproductive cycles were monitored and male-female pairs used only during non-reproductive periods. Sites where ALAN was added ( $n = 17$ ) were paired with control sites (no additional lighting,  $n = 17$ ) of similar reef habitat, structure and depth (1–10 m), allowing for temporal and spatial control between paired sites. Sites within pairs were randomly allocated to the two treatments and were separated by  $>10$  m distance, which corresponded to the distance at which light fully attenuated from the ALAN lighting rig. Control and ALAN sites were subject to light from the moon (depending on phase, elevation and timing) as well as unavoidable low-level skyglow. However, skyglow in this region of the Pacific Ocean is minimal compared to other regions (Falchi et al., 2016).

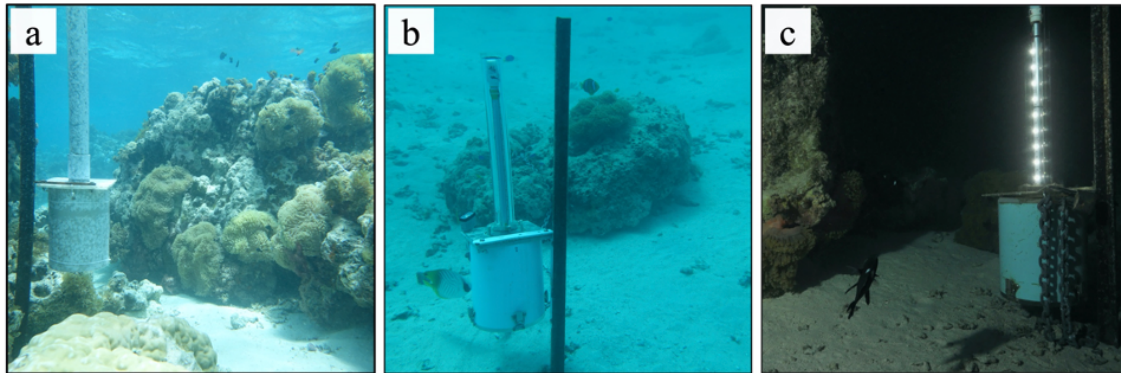


**Figure 3.2** Map of study sites, showing the location of control (white) and ALAN (yellow) anemonefish sites ( $n = 34$ ). ALAN sites were temporally and spatially distanced to avoid light contaminating surrounding control or ALAN sites. Satellite image obtained from Google Maps, available at <https://www.google.com/maps/place/Mo'orea> (Map data: Google, French Polynesia).

### 3.2.2 Experimental Design & Method

The experiment followed a Before–After–Control–Impact (BACI) design, which is considered effective and robust for isolating the influence of the given treatment from natural variability in the environment (Eberhardt, 1976; Green, 1979). At control sites, the female–male pair was exposed to a DUMMY rig (no additional lighting but same physical structure as an ALAN rig; Fig. 3.3a) throughout the experimental period (Fig. 3.4). At ALAN sites, the female–male pair was initially exposed to a DUMMY rig which was then replaced with an ALAN rig that automatically lit up at night via a photosensor system (Fig. 3.3b,c). After visiting ALAN sites to either swap the rig at the start of the ALAN treatment or change

the battery, the corresponding paired control site was also visited. This ensured anemonefish were disturbed (snorkellers duck diving and noise of screwdriver against the stake) to a similar extent across paired control–ALAN sites.



**Figure 3.3** Experimental set-ups, showing (a) a DUMMY rig at a control site, and an ALAN light fixture at an ALAN site (b) during the day and (c) during the night.

The DUMMY rig comprised a PVC housing with a similar external structure to the ALAN light fixtures, but did not emit light. DUMMY fixtures were used so that any potential differences in behaviour between the paired control and ALAN fish pairs were due to exposure to artificial light and not due to the presence of additional substrate or in response to a novel object. ALAN light fixtures comprised a watertight PVC housing containing a 12V battery with three strips of LED lights housed in a transparent cylinder extending from the battery housing. The ALAN light fixtures had a photosensor that meant they became illuminated at dusk; each morning, the ALAN light fixtures were wrapped in dark cloth to check that the LED strips would have been working the previous night. During the study period (in the southern hemisphere summer), sunset ranged from 18:30 to 19:00 and sunrise from 05:00 to 05:40, meaning that fish at the ALAN sites were exposed to 10–11 h of artificial light each night. Both DUMMY and ALAN rigs were secured to a stake placed 1 m from the host anemone. The stake was hammered into the sea floor and the rigs were bolted to the stake. ALAN rigs were chained and padlocked to the substratum. A different 5–7 pairs of control–ALAN sites were tested each month.

The strength of light emitted from lighting rigs can differ depending on the surrounding reef complexity and composition. Therefore, at each ALAN site, light intensities from the lighting rigs were measured (using a SpectroSens2+ sensor; Skye Instruments Ltd.). In addition, two kinds of LED lights were used (referred to henceforth as new LED and old LED). Therefore, light intensity was measured at two standardised locations of differing water clarity and sand colouration: the beach, where there is white sand and clear water, to obtain the highest light intensity possible; and the bay, where the sand is black and the water is murkier, to obtain the lowest light intensity possible. These measurements established that ALAN sites would have light levels at 1 m distance in the following ranges: Old LED: 12 lux (white sand) and 8 lux (black sand); New LED: 26 lux (white sand) and 17 lux (black sand); where lux is a measure of light intensity as perceived by the human eye (lumen per square metre). Control sites, which were located >10 m from the paired ALAN site and >100 m from other human infrastructure and associated sources of artificial light, were exposed only to natural lighting. Light intensity measurements were attempted at the control sites, but the SpectroSens2+ was not accurate enough to record minimal light intensities; light levels were <1 lux.

### 3.2.3 Data Collection

Behavioural data were collected using GoPro video sport cameras (GoPro™, California, US). For each observation period, three video cameras were placed 1 m from the host anemone, positioned strategically to maximise coverage of the female–male pair. Observations were made during the day at three time points at each of the paired control–ALAN sites (Fig. 3.4), however due to unforeseen circumstances (e.g., the fish spawning during the video) the exact days differed:

**BEFORE:** ~ 1 day after the DUMMY rig was introduced (mean±SE: 1.6±0.3 night(s), range: 1–9) .

**IMMEDIATE:** ~ 1 day after the start of the ALAN treatment and an equivalent period at the matched control site ( $1.3 \pm 0.3$  night(s), range: 1–8).

**PROLONGED:** after 14–35 nights of exposure to the ALAN treatment and an equivalent period at the matched control site ( $20.7 \pm 0.9$  nights)

Days	Before d0	Before d1	Treatment d0	Treatment d1	...	Treatment d15-36
<b>CONTROL site</b>						
<b>ALAN site</b>						
<b>Action:</b>	<i>DUMMY secured 1 m away</i>	<b>BEFORE</b> videos taken	<i>Treatment started</i>	<b>IMMEDIATE</b> videos taken	<i>Exposed to treatment for 14-35 nights</i>	<b>PROLONGED</b> videos taken

**Figure 3.4** Methodology throughout the course of the experiment at the paired control–ALAN sites. Key: DUMMY, ALAN on, ALAN off, GoPro, Daytime, Night-time.

The aim was to collect videos on the same day at the control–ALAN paired sites but, due to logistical constraints (e.g., fish spawning early, limitations in equipment, tsunami warnings etc.), videos within pairs could not always be captured on the same day at every time point. Of the 17 paired videos taken BEFORE, six pairs were taken on the same day, one pair was taken at each of 1, 3, 7 and 9 days apart, three pairs were 2 days apart, and four pairs were 4 days apart (mean $\pm$ SE:  $2.5 \pm 0.6$  days apart). Of the 17 paired IMMEDIATE videos, eight pairs were taken on the same day, two pairs were 1 day apart, one pair was taken at each of 2, 7 and 8 days apart, three pairs were 3 days apart, and one pair was not possible due to fish spawning at the paired ALAN site at the time of

video capture (mean±SE: 1.8±0.6 days apart). Of the 17 paired PROLONGED videos, 12 pairs were taken on the same day, two were taken 1 day apart, one was taken 10 days apart, and two pairs were not possible due to spawning (mean±SE: 2.6±0.7 days apart).

#### 3.2.4 Video Processing & Analysis

To avoid observer bias, videos were prepared for analysis by initially visually scanning and then cropping each video so that neither ALAN nor DUMMY rigs were visible throughout the duration of the footage. Videos were renamed with coded numbers and put onto a separate hard drive without any information on site or treatment; videos were scored blind. During this process, original files were cross-referenced to newly named files to ensure files matched.

Prior to analysis, fish in each pair were visually sexed. Within anemonefish groups, a size-based dominance hierarchy persists, with the female being the largest and most dominant fish, the mature male being the second largest fish, and sub-adults/juveniles being smaller, submissive, non-reproductive members of the colony (Buston, 2003b; Buston & Cant, 2006). When the male and female in a pair were close in size, the colouration of the fish was used as a secondary indicator of sex. Social rank is the primary determinant of levels of melanism in *A. chrysopterus*, with the largest fish within an anemone (the dominant female) having greater amounts of black and near-black pigmentation (Militz et al., 2016).

An initial acclimation period of 10 min was given prior to analysing a 5 min period of anemonefish behaviour to allow for fish behaviour to return to baseline following deployment of the cameras (Nanninga et al., 2017). Behaviour was noted using a predetermined ethogram (Table 3.1) using the behavioural analysis software BORIS (Friard & Gamba, 2016). Two behavioural analysis methods

were tested initially: (1) scan sampling of behaviour every 5 s, and (2) continuous recording of the initiation and termination of all behaviours. The interval scoring method was most suitable as it was faster and, following comparison to the continuous method, captured the breadth of behaviours equally well. For each fish (female and male in a pair) and each 5-min observation period (BEFORE, IMMEDIATE, PROLONGED), there was a total of 61 scan samples.

**Table 3.1** Ethogram of orange-fin anemonefish behaviour used in behavioural analysis.

Behaviour Code	Behaviour Type	Description
<i>H</i>	Chasing heterospecific	Fish rapidly swimming directly behind and following a fish of a different species
<i>C</i>	Chasing conspecific	Fish rapidly swimming directly behind and following another anemonefish
<i>V</i>	Vocalisation	Fish creating sound through pharyngeal jaw contraction mechanism
<i>R</i>	Buried in anemone	Fish body nearly completely hidden within the tentacles of anemone (may be able to see head poking through tentacles)
<i>T</i>	Touching anemone	Fish within one body length of anemone
<i>S</i>	Social	Adult female–male pair within one body length of each other
<i>O</i>	Out of sight	Fish leaves window of camera view

Chasing of heterospecifics and conspecifics were combined as an ‘Aggression’ behaviour. Occasions where the fish was buried in the anemone or touching it were combined as ‘Hiding’ behaviour. To obtain a proportion of time for each behavioural group (Aggression, Hiding, Social, Vocalisation), the number of scan samples in which that behaviour was recorded was divided by the total number of scan samples that the focal individual was in sight. If the individual was visible for fewer than 30 scans in a 5-min video, that individual was not included in analyses. For the Social behaviour, both members of the pair had to be in sight for more than 30 scan samples in the video for inclusion in analyses. Behavioural proportion of time values were calculated for the BEFORE, IMMEDIATE and PROLONGED periods for both the male and female fish at each site.



For Aggression and Vocalisation, proportion of time values were extremely low (frequently zero). Therefore, all Aggression and Vocalisation proportion of time values were transformed into categorical data, so each individual fish at each site was either 'non-vocal' or 'vocal' and 'non-aggressive' or 'aggressive' (binary response terms for analysis).

#### *3.2.4.1 Statistical Analysis*

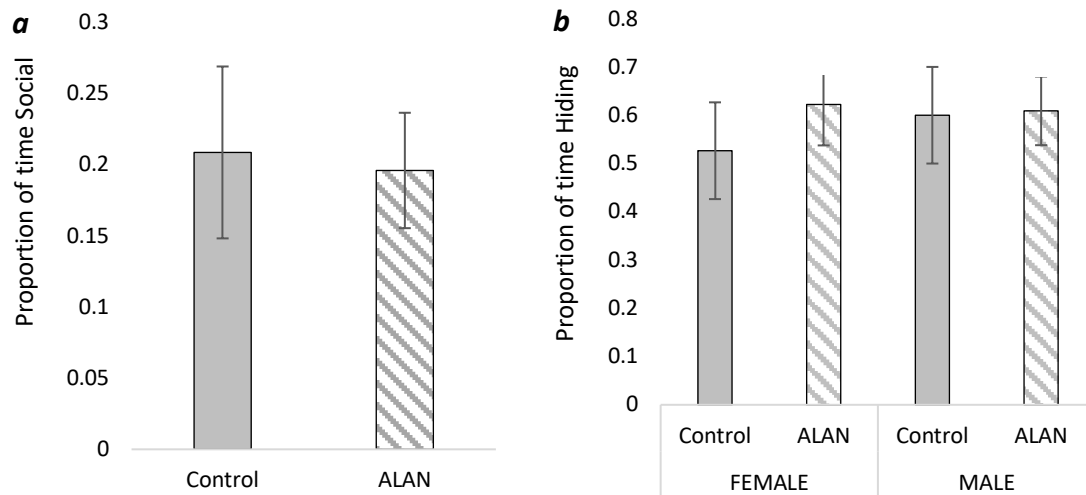
All data were checked for normality, with paired t-tests being used for data sets that fulfilled required assumptions for parametric testing, and non-parametric Wilcoxon signed-rank tests used for data that did not. McNemar's tests were used for contingency tables analysis, with a continuity correction applied, as most of the cell counts in the tables were less than 5. Statistical analyses were conducted in R, using RStudio v.2021.09.1+372 "Ghost Orchid" Release (RStudio Team, 2020), or in SPSS (IBM Corp 2020).

The behavioural data from the BEFORE period in paired control–ALAN sites were first compared to establish whether there were significant differences between the baseline behaviour of fish randomly allocated to the paired control and ALAN sites. Changes in the proportion of time the fish spent performing Social and Hiding behaviour between relevant 5-min observation periods were then compared between treatments. First, to consider the IMMEDIATE impact of ALAN, individual behavioural proportion of time values from the BEFORE period were subtracted from their corresponding value in the IMMEDIATE period for all sites. Second, to consider the PROLONGED impact of ALAN, individual behavioural proportion of time values from the IMMEDIATE period were subtracted from the PROLONGED period across all sites. For the categorical behaviour (Aggression and Vocalisation), McNemar tests were used to compare the occurrence of each behaviour between treatments in either the IMMEDIATE or the PROLONGED period.

### 3.3 Results

#### 3.3.1 BEFORE Period

Baseline Social and Hiding behaviours were not significantly different between the paired control and ALAN treatment sites (paired two-tailed  $t$  tests: Social,  $t(14) = 0.211$ ,  $p = 0.836$ ; Hiding, female:  $t(10) = -0.842$ ,  $p = 0.420$ , male:  $t(10) = -0.063$ ,  $p = 0.951$ ; Fig. 3.5). During the BEFORE period, Aggression and Vocalisation behaviours were also not significantly different in females and males between the control and ALAN treatment groups (Table 3.2; McNemar's test, all  $p$  values = 1).



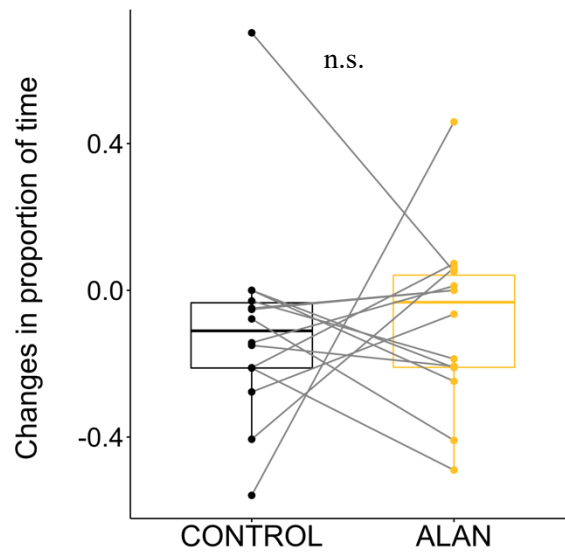
**Figure 3.5** Mean  $\pm$  SE (a) Social (N = 15 paired sites) and (b) Hiding (N = 11 paired sites) behaviour from control and ALAN treatment groups during the BEFORE period.

**Table 3.2.** Frequency of observations with Aggression and Vocalisation behaviours seen during the BEFORE period, according to treatment and sex. Values represent the number of fish that exhibited the relevant behaviour at least once out of all the fish that were observed.

<b>(a) Aggression</b>		
	Control	ALAN
Female	1/11	1/11
Male	3/11	4/11
<b>(b) Vocalisation</b>		
	Control	ALAN
Female	0/11	0/11
Male	0/11	1/11

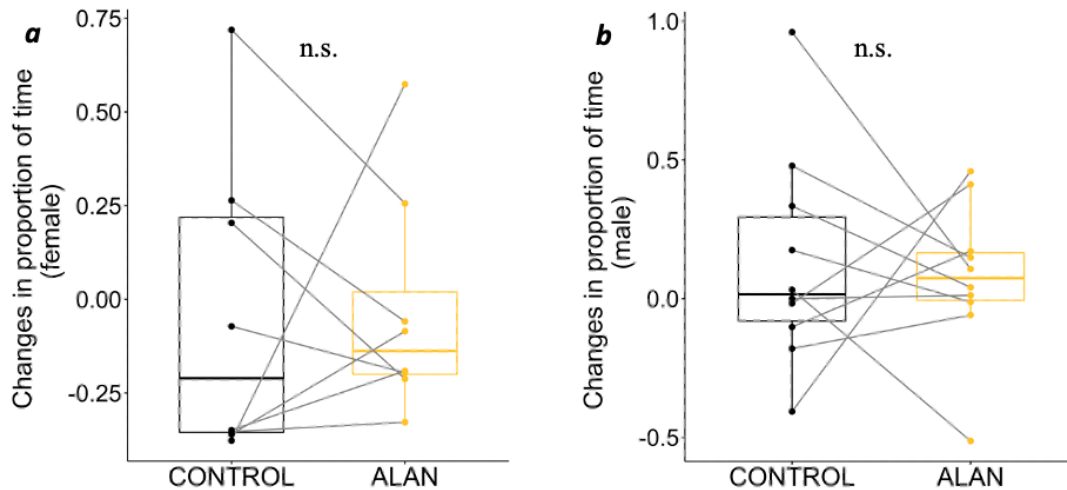
### 3.3.2 IMMEDIATE Period

The change in Social behaviour from the BEFORE to the IMMEDIATE period did not differ significantly between treatments (Wilcoxon signed-rank test:  $z = -0.157$ ,  $p = 0.875$ ; control: median = -0.11 (Interquartile range [IQR]: -0.21, -0.03),  $N = 14$ ; ALAN: median = -0.03 (IQR: -0.21, 0.04),  $N = 14$ ; Fig. 3.6).



**Figure 3.6. Changes in the proportion of time spent being Social in female–male pairs following immediate exposure to treatment.** Each point symbolises a single female–male pair with grey lines corresponding to the matched control and ALAN sites.  $N = 14$ .

The change in female Hiding behaviour from the BEFORE to the IMMEDIATE period did not differ significantly between treatments (Wilcoxon signed-rank test:  $z = -0.280$ ,  $p = 0.779$ ,  $r = -0.07$ ; ALAN: median = -0.14 (IQR: -0.20, 0.02),  $N = 8$ ; control: median = -0.21 (IQR: -0.35, 0.22),  $N = 8$ ; Fig. 3.7a). There was also no significant treatment difference in the change in male Hiding behaviour ( $z = -0.357$ ,  $p = -0.721$ ,  $r = -0.08$ ; ALAN: median = 0.07 (IQR: -0.01, 0.17),  $N = 10$ ; control: median = 0.02 (IQR: -0.08, 0.29),  $N = 10$ ; Fig. 3.7b).



**Figure 3.7. Change in the proportion of time (a) females and (b) males spent Hiding following immediate exposure to treatment.** Each point symbolises one individual with grey lines corresponding to the matched control and ALAN treatment pairs. (a) N = 8, (b) N = 10.

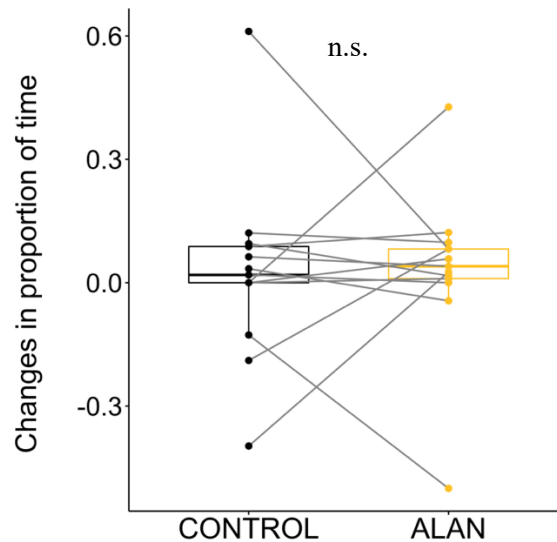
Female and male Aggression did not differ significantly between control and ALAN sites following one night of exposure to treatment (Table 3.3a; McNemar's: females,  $p = 0.371$ ; males,  $p = 1$ ). Similarly, there was no treatment difference in female and male Vocalisation behaviour following one night of exposure to ALAN (Table 3.3b; McNemar's: females,  $p = 1$ ; males,  $p = 1$ ).

**Table 3.3.** Frequency of observations of (a) aggression and (b) vocalisation behaviours seen during the IMMEDIATE period, according to treatment and sex. Values represent the number of fish that exhibited the relevant behaviour at least once out of all the fish that were observed.

<b>(a) Aggression</b>		
	Control	ALAN
Female	1/11	4/11
Male	6/15	7/15
<b>(b) Vocalisation</b>		
	Control	ALAN
Female	0/11	1/11
Male	1/15	1/15

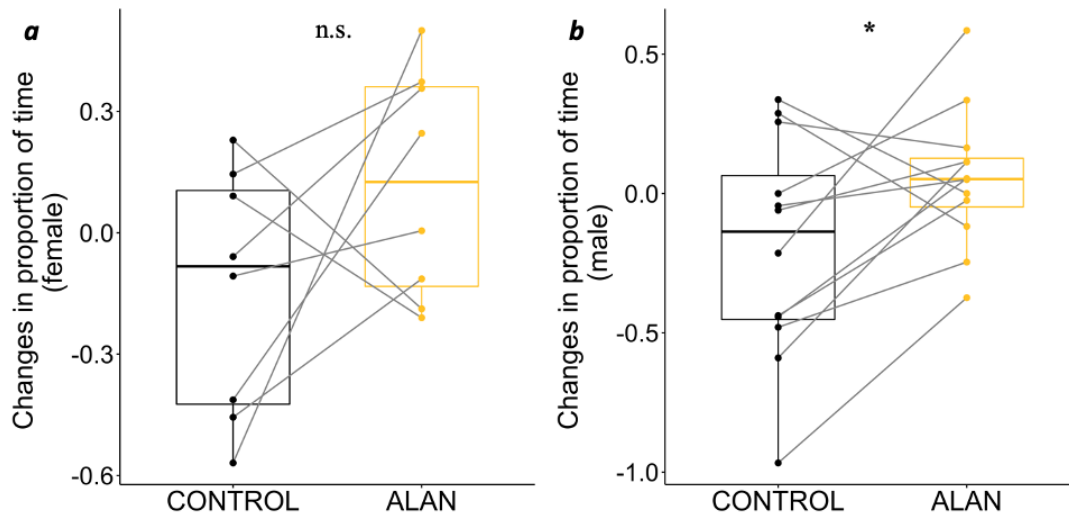
### 3.3.3 PROLONGED Period

The change in Social behaviour from the IMMEDIATE to the PROLONGED period did not differ significantly between treatments (Wilcoxon signed-rank test;  $z = -0.105$ ,  $p = 0.916$ ,  $r = -0.021$ ; control: median = 0.019 (IQR: 0, 0.09),  $N = 13$ ; ALAN: median = 0.040 (IQR: 0.01, 0.08),  $N = 13$ ; Fig. 3.8).



**Figure 3.8. Changes in the proportion of time spent being Social in female–male pairs following longer-term exposure to treatment.** Each point symbolises a single female–male pair with grey lines corresponding to the matched control and ALAN sites.  $N = 13$ .

The change in female Hiding behaviour from the IMMEDIATE to the PROLONGED period did not differ significantly between treatments (Wilcoxon signed-rank test;  $z = -1.260$ ,  $p = 0.208$ ,  $r = -0.315$ ; control: median = -0.083 (IQR: -0.42, 0.10),  $N = 8$ ; ALAN: median = 0.126 (IQR: -0.13, 0.36),  $N = 8$ ; Fig. 3.9a). However, there was a significant treatment difference in the change in male Hiding behaviour ( $z = -1.961$ ,  $p = 0.05$ ,  $r = -0.40$ ; Fig. 3.9b), with ALAN-exposed fish (median = 0.052 (IQR: -0.05, 0.13),  $N = 12$ ) increasing their hiding behaviour more than control fish (median = -0.137 (IQR: -0.45, 0.06),  $N = 12$ ).



**Figure 3.9. Changes in the proportion of time (a) females and (b) males spent Hiding following long-term exposure to treatment.** Each point symbolises one individual with grey lines corresponding to the matched control and ALAN paired sites. \* denotes significant difference. (a) N = 8, (b) N = 12.

Female and male Aggression did not differ significantly between control and ALAN sites following longer-term exposure to treatment (Table 3.4a; McNemar's: females,  $p = 0.134$ ; males,  $p = 0.371$ ). Similarly, there was no significant treatment difference in female and male Vocalisation behaviour following longer-term exposure (Table 3.4b; McNemar's: females,  $p = 1$ ; males,  $p = 1$ ).

**Table 3.4.** Frequency of observations of (a) aggression and (b) vocalisation behaviours seen during the PROLONGED period, according to treatment and sex. Values represent the number of fish that exhibited the relevant behaviour at least once out of all the fish that were observed.

<b>(a) Aggression</b>		
	Control	ALAN
Female	1/10	5/10
Male	2/13	5/13
<b>(b) Vocalisation</b>		
	Control	ALAN
Female	1/14	1/14
Male	1/14	1/14

### 3.4 Discussion

Artificial illumination is increasing every year (Kyba et al., 2017), affecting the natural light and dark cycles of myriad marine organisms (Witherington & Bjorndal, 1991; Kiyofuji & Saitoh, 2004; Merkel, 2010; Santos et al., 2010; Becker et al., 2013; Davies et al., 2015). Anthropogenic stressors, including localised artificial light at night (ALAN) can severely impact species that are site-attached, where movement away from ALAN is not possible (Beldade et al., 2017; Fobert et al., 2019). This study explored the effects of both IMMEDIATE and PROLONGED exposure to ALAN in a site-attached coral reef fish species, the orange-fin anemonefish, *Amphiprion chrysopterus*. The main findings of this study are that IMMEDIATE exposure to ALAN did not significantly affect non-reproductive diurnal behaviour in female or male *A. chrysopterus*, but that PROLONGED exposure significantly altered male diurnal behaviour. This study highlights the importance of assessing potential indirect effects of nocturnal anthropogenic stressors on subsequent diurnal behaviour, and of considering potential intraspecific variation in effects.

#### 3.4.1. Influence of ALAN on Hiding Behaviour

Male orange-fin anemonefish were found to increase diurnal Hiding behaviour following longer-term exposure to ALAN. This differed to natural diurnal behaviour, whereby *Amphiprion* species spend the majority of the day feeding on microscopic prey suspended in the water column (Allen, 1975; Fautin & Allen, 1997; Frédérich et al., 2009). ALAN is known to attract microscopic prey items and larval stages of reef fishes (Fisher & Bellwood, 2002; Simpson et al., 2004, 2011b), so the increase in abundance of nearby prey and the extended hours of light associated with ALAN may result in orange-fin anemonefish extending their feeding window into the night. Feeding involves actively swimming in the water column, resulting in significant energy expenditure in overcoming pressure drag

exerted by water (McKenzie, 2011). In natural environments, *Amphiprion* are known to rest within the tentacles of the anemone at night (Allen 1975; Fautin & Allen 1997). Male orange-fin anemonefish could be residing closer to the anemone during the day due to a decrease in their requirement to feed potentially coupled with a greater need to digest and rest as a result of an increase in nocturnal activity following long-term exposure to ALAN. The need to rest and digest are likely to accumulate over time, perhaps explaining the longer-term change observed.

An alternative explanation for the increase in male hiding behaviour following longer-term exposure to ALAN may be increased antipredator behaviour. Anemonefish are known to be poor swimmers with limited means to defend themselves (other than aggressive biting actions), and so usually retreat to their host anemone when threatened (Mebs, 2009). Previous studies have demonstrated that refuges, such as the host anemone, provide protection from potential predators, currents and strong light, as well as providing a site for foraging and reproduction (Mariscal, 1970; Holbrook & Schmitt, 2002; Almany, 2004; Johansen et al., 2008; Kerry & Bellwood, 2012). Sea anemones (Class Anthozoa) have venomous nematocysts and mucous secretions used for both prey acquisition and also to deter potential predators (Mebs, 2009). The skin of *Amphiprion* species is coated in a mucus layer protecting them from these harmful venoms (Caspers 1939), thus, sea anemones (e.g., the orange-fin anemonefish host, *Heractis magnifica*) act as a safe haven for these vulnerable fish (Mariscal, 1970). Artificial light can attract predators at night (Becker et al., 2013), so the increased diurnal hiding behaviour seen here could be a carry-over effect from an increased nocturnal antipredator response. Fish have to trade-off the risk of predation with the cost of using refugia, explained by the optimal hiding time model (Martín & Lopez, 2015). Male fish may stay in the safety of the host anemone after predators have left, limiting subsequent foraging and feeding opportunities (Sih, 1992). This may mean that nocturnal feeding is insufficient to compensate for this decrease in diurnal feeding. Thus, future ALAN studies could



explore changes in fish body condition with prolonged exposure to ALAN (Bolton et al., 2017).

Nocturnal fish behaviour is largely unexplored (but see: Fisher & Bellwood, 2003), and so to test potential explanatory theories, nocturnal orange-fin anemonefish behaviour needs to be assessed under ALAN to ascertain whether (1) foraging activity extends into the night, and (2) whether there is an increase in nocturnal anti-predator behaviour. If these theories are supported, exposure to long-term ALAN could result in orange-fin anemonefish potentially switching to nocturnalism, with exposure to light potentially reversing the natural cycle of foraging during the day and resting at night. A switch in timing of activity has previously been documented in *Amphiprion ocellaris* where nocturnal larvae switch to become diurnal adults (Schalm et al., 2021). However, this shift in behaviour is associated with development and not due to exposure to a stressor. Therefore, future research should assess whether exposure to ALAN over many months/years results in a more permanent switch to nocturnalism in orange-fin anemonefish.

#### 3.4.2 Consequences for the Anemone of Increased Hiding by Anemonefish

Increased association between the male anemonefish and the host anemone could influence anemone health. Association with orange-fin anemonefish benefits the host anemone (*Heractis magnifica*) considering orange-fin anemonefish: (1) help to protect the anemone from predators (Godwin & Fautin 1992; Porat & Chadwick-Furman 2004), and (2) provide the host anemone with nutrients (e.g., ammonia, sulphur and phosphorous; Porat & Chadwick-Furman, 2004, 2005). More recently, *Amphiprion* species have been shown to increase water flow near to the host anemone (Szczebak et al., 2013). Szczebak et al. (2013) found that *Amphiprion bicinctus* (Rüppel, 1930) spend a significant portion of the night in some form of motion. Szczebak et al. (2013) simultaneously found

increased net oxygen uptake in *A. bicinctus* and the sea anemone partners and suggested that this association enhanced water flow allowing for increased gaseous exchange with the host anemone. Similarly, Goldshmid et al. (2004) found that sleep-swimming damselfishes beat their fins at twice as fast at night, increasing water flow near to anemones. Further work is needed to ascertain whether orange-fin anemonefish provide anemonefish-induced flow modulation around *H. magnifica*. If so, increased hiding behaviour during the day by male *A. chrysopterus* observed here may directly benefit the host anemone. Conversely, if orange-fin anemonefish feeding behaviour is stimulated at night under exposure to ALAN, resulting in a greater time spent away from the anemone, then the increased hiding during the day seen here may result in a net neutral effect on sea anemone health. This further highlights the need to assess orange-fin anemonefish behaviour at night, under ALAN, as well as assessing growth and health of their symbiotic host.

#### 3.4.3 Recovery Post-exposure

There is increasing awareness of the effects of both short- and longer-term exposure to stressors, as explored in this study, in a range of taxa (Fobert et al., 2019, 2021; O'Connor et al., 2019; Mills et al., 2020; Schligler et al., 2021). However, recent research has explored the scope for recovery after a stressor is removed (Fobert et al., 2019). Assessing the influence of ALAN on egg hatching in *Amphiprion ocellaris*, Fobert et al. (2019) found that eggs from clutches reared at ALAN sites had a hatch rate of 0% during the exposure period, but that during a recovery period of 60 days post-exposure, there was no significant difference in the proportion of eggs that hatched between ALAN and control groups. This suggests that negative effects on *Amphiprion* eggs seen during exposure to ALAN can be reversed. The methods used in the study could be extended to test the effect of the removal of ALAN on the behaviour of male *A. chrysopterus*. This would involve taking videos after the exposure period, such as: day 1 and day 30 post removal of exposure (mirroring that of the treatment period). This would

allow for a broader understanding of the resilience of *A. chrysopterus* to ALAN, allowing for the development of appropriate mitigation strategies and aiding in legislation choices. For example, if male *A. chrysopterus* hiding behaviour quickly returned to baseline following removal of artificial lights, potential short-term 'breaks' in artificial light could enhance resilience of *A. chrysopterus* to stressors.

#### 3.4.4 Minimally Influenced Behaviours

The results found here highlight that both IMMEDIATE and PROLONGED exposure to ALAN have a minimal effect on adult female and male orange-fin anemonefish diurnal behaviours, with aggression, social and vocalisation behaviours not differing significantly across treatments. The non-significant effect of ALAN on aggression may be explained by the theory that orange-fin anemonefish have an increased need to digest and rest following increased foraging at night. It is widely accepted that aggressive acts involve energetically costly behaviours (Vøllestad & Quinn, 2003; Martorell-Barceló et al., 2020), such as chasing that require high-speed swimming. Therefore, ALAN-exposed fish may be too satiated or lack sufficient reserves to perform energetically costly acts. Additionally, aggression may not be affected by ALAN due to a potential increased nocturnal antipredator response carrying-over into the following day. Unlike the results found here, Mills et al. (2020) found that *A. chrysopterus* aggressive behaviours were triggered in response to boat noise, with this increase being due to a rise in hormone levels. In this present study, it is unlikely that hormone levels were raised considering the stressor of ALAN was effectively removed during the day. This could help to explain a non-significant effect in social behaviour too, considering social behaviours are largely hormonally modulated (Oliveira, 2009). Orange-fin anemonefish rarely vocalise unless under agonistic scenarios (as found in **Chapter 2**), and considering aggression and vocalisation is linked, it is therefore unsurprising that ALAN did not significantly affect either behaviour.

The response of longer-term exposure to ALAN on hiding behaviour differed between the sexes. The lack of a significant effect of ALAN on female hiding may be due to an insufficient sample size (there was a non-significant trend; Fig. 3.9), rather than there being a real difference in response to ALAN between the sexes. However, the difference in effect might be due to sex differences in stress response. The hypothalamus-pituitary-interrenal (HPI) axis plays an important role in orchestrating the response of different teleost species to a range of stressors (Wendelaar Bonga, 1997; Flik et al., 2006). This axis ranks as the most prevalent and evolutionarily conserved adaptation to stress and, when activated, glucocorticoid is secreted, increasing blood glucose and resulting in enhanced ventilation rate and cardiovascular activity, all necessary for the fight-or-flight response (Sapolsky, 1990; Sapolsky et al., 2000; Hawlena & Schmitz, 2010; Clinchy et al., 2013; Vinterstare et al., 2021). Previous studies have found that responses by females to stress are often lower than those of males, suggested to be linked to processes relating to caregiving and attachment that potentially downregulate the HPI axis, reducing the scope and increasing the threshold for stress responses in females (Taylor et al., 2000; Afonso et al., 2003; Donelan & Trussell, 2020). However, this theory is limited in explaining the results of this study considering female orange-fin anemonefish are not the predominant caregivers in the parental pair; in anemonefish the males are responsible for fanning and protecting the eggs (Moyer & Bell 1976). Nonetheless, female orange-fin anemonefish have the role of producing and laying high-quality, lipid-filled eggs – a metabolically demanding activity (Mustonen et al., 2002; Glebe & Leggett, 2011; Nielsen et al., 2022). Stress exposure has been shown to decrease egg size in brown (*Salmo trutta*) and rainbow (*Oncorhynchus mykiss*) trout, suggesting that although high-stress tolerance aids in escaping acute threats, there can be potential associated costs to egg quality (Campbell et al., 1994). Sexual dimorphism in the hiding behaviour response after long-term exposure to ALAN seen in this study may relate to differences in stress response between the two sexes. A recent study highlighted that when exposed to stress, Trinidadian guppy (*Poecilia reticulata*) females had a lower cortisol release than males (Chouinard-Thuly et al., 2018). Future work should aim to measure cortisol

levels in female and male *A. chrysopterus* during both short- and long-term exposure to ALAN, to assess sexual dimorphism in stress response at the hormonal level.

In contrast to the findings of this study, previous work has highlighted significant effects of ALAN on orange-fin anemonefish life-history traits, in particular, early life stages. An *in situ* experiment, at the same reefs used here, found that both survival and growth in wild juvenile orange-fin anemonefish exposed to long-term ALAN (18–23 months) decreased significantly (36% survival and 44% growth) compared to that of juveniles exposed to natural moonlight (Schligler et al., 2021). Likewise, Fobert et al. (2019) found significant effects of exposure to ALAN in the laboratory on egg hatching in *Amphiprion ocellaris*, with all eggs incubated under exposure to ALAN failing to hatch. This highlights the sensitivity of early life stages of *Amphiprion* species to light pollution, but indicates that in this present study, either: (1) fish were not exposed to ALAN for sufficient time (14–35 nights) to yield significant results, and/or (2) adults are more resilient to exposure to ALAN. Alternatively, diurnal behaviour may not be the best indicator of the impact of exposure to ALAN, with life-history traits (e.g., reproduction, growth, longevity) potentially being more sensitive and susceptible to the negative impacts of ALAN. In contrast to other vertebrates, adult fish have different sensitivities to light compared to juveniles and larvae since the retina in the eye grows continually throughout life, with new neurons continually being added and/or existing tissue being stretched (Fernald, 1988). Future work should assess the effect of stressors across all life stages using comparable metrics to ascertain vulnerable stages in different species and across contrasting life histories.

### 3.4.5 Sensitisation vs Habituation

Animals may respond to repeated exposure to stressors by either becoming increasingly sensitive to the stressor (sensitisation), with heightened responses, or becoming increasingly acclimated to it (habituation), with baseline behaviours returning to normal (Romero, 2004; Bejder et al., 2009; Radford et al., 2016). Short-term exposure to an alternative stressor, motorboat-noise playback, influenced behaviour in orange-fin anemonefish, but long-term exposure led to negligible changes in behavioural and hormonal response (Mills et al., 2020). Goldfish (*Carassius auratus*; a hearing specialist) have also been found to become habituated to noise pollution, such that only short-term exposure (~10 minutes) significantly raises plasma cortisol (Smith et al., 2004). These studies on noise pollution contrast with the findings of this study, which found significant effects in male orange-fin anemonefish after longer-term exposure to ALAN, suggesting sensitisation. A previous study on the effects of ALAN on orange-fin anemonefish larvae found a significant negative response to ALAN following long-term exposure (Schligler et al., 2021). This suggests that, unlike with noise pollution, light pollution effects may accumulate over time. However, Perkin et al. (2014) found that the fry of cutthroat trout (*Oncorhynchus clarkii*) exposed to ALAN had delayed dispersal and lower cortisol release rate. Perkin et al. (2014) suggested that this could indicate acclimation to ALAN, in contrast with the results of Schligler et al. (2021) and this study. To date, light pollution studies have predominantly focused on two ALAN treatments, ALAN vs a no ALAN control, with few studies assessing a dose (intensity)-dependent response (Gaston et al., 2015). Future studies could valuably assess effects of light pollution over short- and long-term exposure, to enable thresholds and intensity-dependent relationships to be determined.

#### 3.4.6. Multistressor Effects

When combined, two or more stressors can have: (1) additive, (2) compensatory, (3) independent or (4) antagonistic effects (Parrott & Sprague 1993). The effects of physical and chemical stressors has been well-documented in the literature (Birk et al., 2020; Oliveira et al., 2014), with particular emphasis on combined chemical and temperature effects (Hallare et al., 2005; Moe et al., 2013; Ferreira et al., 2016). However, little is known about the combined effects of local stressors such as light and noise pollution, eutrophication and sedimentation. Due to the field-based approach of this experiment, and associated lack of control over extraneous variables, there is potential for other anthropogenic stressors to influence fish behaviour, including nitrogen runoff (Donovan et al., 2020), changing temperature regimes (Perkin et al., 2011, 2014), human disturbance (Davenport, 2006) and noise pollution (Nedelec et al., 2016, 2017; Mills et al., 2020; Leduc et al., 2021). However, the influence of these effects was minimised through the paired design of this study, controlling for location and thus local variations in exposure to other stressors. The approach of this study is more realistic in considering *in situ* exposure to ALAN alongside other natural and anthropogenic stressors, as it focuses on organisms living in the wild but within close proximity to humans. Nonetheless, future *in situ* studies could explore variation in the levels of other localised stressors, such as motorboat noise, to test ALAN in combination and explore multi-stressor effects.

#### 3.4.7 Type of Light

In this study two types of LED lights were used (old and new LEDs), with light intensities of 8-12 lux and 17–26 lux, respectively. Although these ranges do not overlap, they are both considerably lower than light intensities found in strongly lit areas (lux values at ports and harbours can be as high as 150–200 lux; Bolton et al. 2017). Therefore, effects of ALAN on *Amphiprion spp.* living closer to urbanised coastlines may be greater than seen in this study. Further analyses

could test for differences in the effect of the new vs old LEDs, but here, splitting the data into smaller cohorts based on the ranges of light levels would result in sample sizes too small for valuable analysis.

While the intensity of ALAN is important, so too is the type of light used. For example, swimming speed in juvenile rudd (*Scardinius erythrophthalmus*, Cyprinidae) was found to be significantly higher under a halogen light of lower light intensity (5.4 lux) than under a high-pressure sodium light with greater light intensity (8.2 lux) (Talanda et al., 2018). Therefore, the type of light source emitting ALAN may also influence the level of disruption to fish behaviour. Additionally, previous research has highlighted that fish may respond differently to varying colours/wavelengths of light. For example, Lin et al., (2021) found that swimming activity in the cyprinid, *Ptychobarbus kaznakovi*, was greatest under yellow and red light when compared with blue and green. These results indicate that light of varying wavelengths can differentially affect fish behaviour. In this present study, only one type of light was used (white LED), therefore, future work could assess the effects of varying wavelengths of light from differing sources. This further would aid in reducing negative impacts of ALAN on coral reef fishes by allowing the development of mitigation strategies in which wavelengths of light with the least effect are used at locations in close proximity to reefs. Additionally, light could be used as a possible attractant to help in guiding or excluding fish from particular high-stressor locations (Lin et al., 2021).



Chapter 4:  
General Discussion

## 4.1 Discoveries

By studying orange-fin anemonefish behaviour and acoustic communication *in situ*, I have, to my knowledge, produced the first body of work showcasing the natural variation in orange-fin anemonefish vocal signals, highlighting the complexities within vocalisations, and exploring how vocal behaviours can be influenced by a localised coral reef stressor: artificial light at night (ALAN). I have highlighted how orange-fin anemonefish vocalise infrequently unless confronted with a threat, shown how these vocalisations have sex-dependent traits, and identified intricacies within specific vocalisations that are more varied than previously documented in the literature. Assessing behavioural change in response to a pervasive coral reef pollutant, I have highlighted how ALAN disproportionately influences males over females, with a significant influence seen only in male behaviour following long-term exposure. Through the findings displayed in this thesis, I have highlighted how natural populations of anemonefish inhabiting coral reefs can act as excellent model systems for the study of baseline behaviour and vocalisation, allowing testing of impacts of specific stressors in the rapidly changing environments of coral reefs.

Communication in fish has been known of since the time of Aristotle (350 BC; Aristotle et al., 1965). However, the environments in which fish live and communicate are changing at an accelerating rate; primarily linked to the rise in anthropogenic activities. In the past 100 years, the human population has quadrupled (UN, 2019), with scientists now referring to this most recent period of time as the 'Anthropocene' in which Earth's climate and ecosystems have significantly been impacted by human activity (Steffen et al., 2007). One-quarter of the human population lives within 100 km of the world's coastlines (Small & Nicholls 2003), resulting in significant localised stressors on coastal ecosystems worldwide. More specifically, localised stressors on coral reefs include thermal-induced bleaching (Beldade et al., 2017), eutrophication (Lesser, 2021), sedimentation (Moustaka et al., 2018), noise pollution (Mills et al., 2020) and light

pollution (O'Connor et al., 2019; Fobert et al., 2021; Schligler et al., 2021), which have all been shown to have a significant negative influence on the behaviours and populations of resident reef fish species. Therefore, it is important to understand changes to the breadth and complexity of baseline fish behaviour and communication within these increasingly stressful environments.

Having identified underlying complexity in anemonefish acoustic signalling, as presented in **Chapter 2** from an *in situ* study, I propose anemonefish as a valuable model species for the future collection of wild behaviour and vocalisations on coral reefs. Through the use of simple, inexpensive, and readily available equipment (such as a mirror and a GoPro), my study provides new understanding of complexity in acoustic communication in fishes on coral reefs.

In **Chapter 3**, I found that exposure to long-term light pollution at night can carry over into the day, disproportionately affecting the diurnal behaviour of male orange-fin anemonefish, but with minimal effects on females. This is particularly important, as coral reefs are marine regions located in close-proximity to humans, with significant associated light pollution (Grimm et al., 2008). Orange-fin anemonefish are site-attached, and so are limited in their ability to relocate away from stressors such as ALAN. However, unlike other more global anthropogenic stressors, such as ocean acidification and ocean warming, with relatively little effort the stressor of light pollution is relatively easy to mitigate with immediate effect.

Marine management in the last few decades has focused on managing fisheries, pollution, offshore development and mineral extraction (Halpern & Warner, 2002; Lester et al., 2009). Marine protected areas (MPAs) are a useful tool in managing oceans (Lester et al., 2009), however, more recent research has highlighted how even regions with the highest level of protection are exposed to

artificial light. In 2012, 35% of all MPAs were exposed to artificial light with 9% of MPAs with the highest protection status (IUCN Category I) experiencing increased levels of light intensity (Davies et al., 2015). I would propose that the issue of light pollution are addressed in future management strategies, and I hope that the methods developed and preliminary findings in this thesis will aid in this endeavour. Light can infiltrate past management boundaries into protected areas, and the ecological impacts of ALAN in marine environments must be assessed to appropriately inform and justify mitigation efforts and legislation.

## 4.2 Future Applications

### 4.2.1 Anemonefish as a Study System

In this thesis, I focused on orange-fin anemonefish as a study species, enabling assessment of both complexity in anemonefish communication and the influence of an environmental stressor on anemonefish behaviour. In **Chapter 1**, I detailed how orange-fin anemonefish are useful for research, with key traits including: a site-attached nature, complex yet defined social structure and well-established life-history (section 1.3). Having undertaken this research, I expand further on the logistical benefits of using orange-fin anemonefish as a study species.

Logistically, research on anemonefish is optimal considering their coral reef habitat is characterised as being shallow, light and relatively easy to access (e.g., via shore dives). Additionally, anemonefish habitats (i.e., the host anemone) vary naturally in size and often occur as discrete patches (Thompson et al., 2007; Wong & Buston, 2013). Through the introduction of equipment ranging from a simple mirror (in **Chapter 2**) to the more specialist dummy and ALAN light structures (in **Chapter 3**), I have highlighted the ease of which the anemonefish habitat can be manipulated to enable experimental testing of defined research questions (Fig. 4.1). Moreover, it is important to note that since

anemones are not firmly attached to the benthos, it would be relatively easy to further manipulate the environment by repositioning the anemonefish habitat in the field. This allows for wide-ranging ecological experiments to be executed (e.g., Wong, 2010). In addition, the ease of collection of the anemone and associated anemonefish means that the discrete habitat patch can be relocated to a laboratory. This is particularly important for undertaking detailed behavioural experiments, where control over extraneous variables is required. Therefore, I propose orange-fin anemonefish (and other anemonefish species sharing similar life-history traits) as an appropriate and tractable model system to assess vocal and behavioural responses to a changing ocean.



**Figure 4.1:** Photograph showcasing the ease at which anemonefish habitats can be manipulated i.e. introduction of a dummy lighting rig via a stake secured to the benthos to the left of the image, and two cameras either side of the focal anemonefish site in the middle of the image. Anemonefish habitats are shallow and bright, so sites can be reached via free-diving, negating the need for more expensive SCUBA equipment/qualifications. Image: Anne Haguenaer.

#### 4.2.2 Value of *In Situ* Studies

In this thesis, I developed methodologies for measuring various *in situ* behaviours of orange-fin anemonefish. I found that orange-fin anemonefish rarely vocalise without specific motivation, as presented in **Chapter 3**. However, in **Chapter 2**, I demonstrate how effective a mirror is in inducing vocal behaviour. Therefore, I would suggest that future studies assessing the effects of ALAN on vocal communication and behaviour in territorial coral reef fish should aim to incorporate the use of a mirror. As such, a combination of methodologies from both chapters would aid in the understanding of the influence of ALAN on both: 1) the response of the focal fish to a perceived rival fish and 2) the acoustic metrics of vocal communication, across both short- and long-term exposure.

Furthermore, I propose that this methodology can be applied in future studies to aid in understanding the extent to which other stressors (e.g., noise pollution) may impact anemonefish communication and behaviour. The research presented here suggests light pollution has a limited influence on adult diurnal behaviour, but other stressors may have more profound impacts. Additionally, this methodology could be implemented to assess changes in behaviour over time. For example, here I found minimal effect of light pollution, but what might happen if the surrounding coral reef habitat becomes degraded due to a severe bleaching event, or a new cruise liner port is built in close-proximity to anemonefish sites? The methodologies developed here could also be used to measure the value of any mitigation efforts, e.g., assessing the effect on fish communication and behaviour of an increase in water quality resulting from legislation reducing the widespread use of fertilisers.

## 4.3 Future Directions

### 4.3.1 Sex Differences in Behaviour

In this thesis, I found sex differences in pulse peak frequencies (**Chapter 2**) and in impacts of light pollution (**Chapter 3**) that would not have been detected if analysing all the data pooled. This is not unusual, considering sex-based divergence in response to stressors has been found across a diverse array of vertebrates (Campbell et al., 2021). Previous research has found that female fish can have weaker immune responses (Shepherd et al., 2012) and greater disease susceptibility (Dong et al., 2017) compared to male fish, in direct contrast to mammals. Therefore, in future studies aiming to assess vocal behaviour and communication, and test for the impacts of stressors, I advocate that male and female responses should be analysed separately. This allows for 1) underlying sex-based divergence in response to be identified, and 2) appropriately informed sex-dependent mitigation tactics to be established.

### 4.3.2 Technology

Few studies have collected and analysed vocalisations obtained in the wild due to limitations in pinpointing individuals when they are vocalising. This present study overcomes this limitation since orange-fin anemonefish vocalise in an obvious manner with specific motor actions indicative of vocal communication, allowing individual vocalisations to be attributed to individual fish from video. However, the majority of coral reef fish do not vocalise in visibly obvious ways, meaning that locating vocalising fish can be extremely difficult. Thus, research on fish vocal communication has typically focused on fishes that are stationary and site-specific, for example toadfish that enter and call from shelters, allowing vocalisations to be assigned individual fish (Amorim et al., 2009, 2010). Further advances in video-audio technology to pinpoint vocalising fish are required to

adapt this approach to assess impacts of localised stressors on vocal behaviour in a wider range of coral reef fishes.

To pinpoint the location of whales, cetacean biologists often use hydrophone arrays. These arrays are usually bulky, expensive and require specialised software for post-hoc analysis. However, an alternative method involving two hydrophones divided by a soundproof disc allows directionality in sound to be assessed whilst on the move (Filatova et al., 2006). Here the sound from each hydrophone feeds into a different channel of a stereo recording system, which is broadcast to the ears of the operator via headphones (Filatova et al., 2006). A device adopting similar ideas but on a smaller, fine-tuned scale could be hugely beneficial for exploring directionality of sounds on a reef in real-time, enabling sounds to be identified to species, and even individual fish, irrespective of whether they are site-specific or free-roaming. With this technological advance, a broader cross-community understanding of reef fish communication might become more possible.

#### 4.4 Concluding Remarks

The world is changing at an unprecedented rate. It is crucial to understand the effects of this change on the myriad marine organisms inhabiting the world's oceans. As coral reef ecosystems become increasingly disturbed and degraded, understanding baseline fish behaviour and the influence of stressors will help build a more holistic understanding of coral reefs and their potential resilience to future change. Here, I have developed a case study that contributes to this understanding, providing methodologies that could be adapted not only for other stressors, but also used to assess the value of different mitigation measures. These studies will support targeted management of local threats, giving coral reefs greater resilience against global stressors in this rapidly-changing ocean.



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