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NEURAL AND ECOLOGICAL BASIS OF PAIR BONDING IN BUTTERFLYFISHES

(F: CHAETODONTIDAE)

Thesis submitted by:

Jessica Ploetz Nowicki

(BA, GradDipResMeth)

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In the ARC Centre of Excellence for Coral Reef Studies

James Cook University

Townsville, Queensland, Australia

Dedication

To my family and my pair bond, Darren James Coker

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Statement of the contribution of others

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Chapter 2:

- Jessica Nowicki: concept of study, data collection, data analysis, writing of manuscript
- Morgan Pratchett: concept of study, writing of manuscript
- Stefan Walker: concept of study
- Lauren O'Connell: writing of manuscript
- Andrew Hoey: writing of manuscript
- Darren Coker: data collection, data analysis

Chapter 3:

- Jessica Nowicki: concept of study, data collection, data analysis, writing of manuscript
- Morgan Pratchett: concept of study, writing of manuscript
- Stefan Walker: concept of study, data analysis
- Lauren O'Connell: concept of study, data analysis, writing of manuscript
- Darren Coker: data collection

Chapter 4:

- Jessica Nowicki: concept of study, data collection, data analysis, writing of manuscript
- Morgan Pratchett: concept of study, writing of manuscript
- Stefan Walker: concept of study, data analysis
- Andrew Hoey: writing of manuscript
- Darren Coker: data collection
- Katia Nicolet: data collection, data analysis

November 6, 2016

Signature Date

Declaration of permits and ethics

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *James Cook University Policy on Experimentation Ethics,*
Standard Practices and Guidelines (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001).

All research pertaining to this thesis was carried out under Great Barrier Reef Marine Park Authorization Permits Nos. G10/33239.1, G13/35909, G13/35909.1, and G14/37213.1; General fisheries permit No. 170251, and JCU Ethics Approval No. A1874.

November 6, 2016

Signature Date

Abstract

Pair bonding has independently evolved in all major vertebrate lineages, where it represents a major defining feature of species-specific social structure. As such, proximate neural reasons for *how,* and adaptive reasons for *why* pair bonding occurs are fairly well established, at least for mammals and birds. In these later vertebrates, particularly mammals, there are four integral neurochemical systems involved: oxytocin, arginine vasotocin, dopamine, and opioid systems. Both oxytocin and arginine vasotocin systems facilitate partner attachment, presumably by mediating social memory. Meanwhile, dopamine and opioid systems appear to facilitate partner attachment by mediating partner reward learning and associated motivation/positive hedonics, respectively. Much less is known about the underlying neural or adaptive basis of pair bonding among reptiles, amphibians, and fishes**.** This is nonetheless very important, mainly because these lineages possess specific qualities, such as the general lack of bi-parental care, that facilitate improved understanding neurobiological systems that independently underpin pair bonding. Moreover, these lineages provide insight into the early evolution and subsequent evolutionary history of vertebrate pair bonding.

The overall objective of my thesis was to investigate the i) underlying neurobiological basis and ii) ecological benefits of pair bonding in fishes, and specifically, among coral reef butterflyfishes (f: Chaetodontidae). As a first step, I sought to establish a novel butterflyfish model system for conducting integrative, comparative and experimental neural research. By undertaking extensive *in situ* behavioural observations on wild fishes, I show that pair bonding vs. non-pair bonding sociality varies markedly among adults of *Chaetodon lunulatus* (84% are pair bonding, whereas 16% are solitary), and among six congeners (84% of *C. lunulatus,* 78% of *C. baronessa,* and 71% of *C. vagabundus* adults are pair bonded, whereas 88% of *C. rainfordi,* 90% of *C. plebeius,* and 80% of *C. trifascialis* adults are solitary). Interestingly, several key attributes, including parental care, do not co-vary with these species differences in sociality. I also show that an ecologically relevant character of *Chaetodon* pair bonding, namely preferential affiliation with partner, is reliably elicited in *C. lunulatus* males using the laboratory "two-choice proximity" assay. When given a choice to affiliate with either their partner or a non-partner female conspecific, the majority of males spent on average 54/60min affiliating with their partner, and only 8/60min affiliating with a non-partner female. These findings reaffirm previous assumptions of the sociality of these species, and validate that the proposed butterflyfish systems are amenable for undertaking highly controlled comparative, and reliable experimental research into fish pair boding.

I then used the established *C. lunulatus* model system for conducting integrative neural research, testing the hypothesis that regulatory neuro-chemical and –anatomical

substrates may be similar to the mammalian model, *Microtus ochrogaster*. Peripheral administration of isotocin (IT, teleost homologue of oxytocin), arginine vasotocin (AVT, teleost homologue of arginine vasopressin) V1a receptor antagonists attenuates partner preference in males, indicating their functional involvement in pair bonding; however, administering dopamine D1 or mu-opioid receptor antagonists has no significant effect. Comparisons of gene expression of ITR, V1aR, D1R, D2R, and MORs within eight brain regions between pair bonded and solitary individuals showed that for females, differences in IT and V1a nonapeptide receptor expression within the lateral septum-like region (the ventral and lateral regions of the ventral telencephalon, Vv/Vl) is associated with differences in pairing phenotype. It further revealed that for both sexes, differences in dopamine D1, D2, and mu-opioid receptor expression within several regions of the mesolimbic reward system, including the striatum-like region (the central nucleus of the ventral telencephalon, Vc), is associated with differences in pairing phenotype.

Finally, to explore the ecological basis of butterflyfish pair bonding, I tested the assisted resource defence hypothesis (ARDH) for pair bonding in two strongly pair bonding *Chaetodon* species. *In situ* observations of wild individuals suggest that paired individuals assist their partners while defending feeding territories in a species-specific manner, such that *C. lunulatus* displays mutual partner assistance, whereas *C. baronessa* displays male-prioritized partner assistance. In both species, partner assistance appears to confer gains in feeding and energy reserves to partners over their solitary counterparts. Experimentally inducing new partnerships *in situ* immediately evoked marked declines in relations between partners of the new pair and between the new pair and their neighbouring pairs, leading to severe declines in feeding rate that eventually recovered with subsequent partner fidelity. Taken together, these findings corroborate with previous findings in butterflyfishes, further supporting ARDH for pair bonding in these organisms, and furthermore suggests that partner fidelity is critical for promoting assisted resource defence.

Overall, the results of this thesis demonstrate that in butterflyfishes, nonapeptide, dopamine, and opioid systems acting within specific nodes of the vertebrate social decision making network regulate pair bonding, in order to provide social assistance during defense of food resources. Based on the broader comparison of these results with those of mammals and birds, I furthermore conclude that in at least very selective cases, the convergence of pair bonding across exceptionally wide evolutionary distances is a consequence of pair bonding repeatedly serving an analogous ecological function through the repeated co-option of homologous neural structures. In order to determine the extent to which this has occurred, however, complementary studies in more vertebrates (most urgently amphibians and reptiles) are now needed.

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Chapter 1: General introduction

The selective (if not exclusive) affiliation between two individuals within wild species often evokes strong emotive responses by human observers, and has obvious connotations for understanding human sociality and behaviour (Reichard and Boesch, 2003; Young and Wang, 2004; Freeman and Young, 2016). Accordingly, there has been extensive research on species that form pairs, as well as the causes and consequences of pair bonding (Reichard and Boesch, 2003; McGraw et al., 2010; Freeman and Young, 2013; Lukas and Clutton-Brock, 2013). Initial research generally focussed on documenting the prevalence of pair bonding within different groups of species, which was well established for some groups in the early 1900s (e.g., birds: Lack, 1940), but continues to be resolved for other groups (e.g., coral reef fishes: Brandl and Bellwood 2014). Observed differences in the incidence of pairing inevitably leads to tests of the endurance or permanency of pair bonds, as well as exploration of the ecological or adaptive benefits accrued from selective affiliations between paired individuals (Black et al., 2001, 2014). Among pair forming birds, life-long affiliations between recognisable (e.g., tagged) individuals as well as the reluctance to form new affiliations following the loss of a mate were viewed as strong evidence for exclusive "monogamous" mating between paired individuals (Lack 1968). However, genetic analyses of resulting progeny are revealing very high incidence of extra-pair copulations (reviewed by Westneat et al. 1990; Westneat and Stewart, 2003; Wolff and MacDonald, 2004; Solomon and Keane, 2007). Recent research is increasingly focussing on establishing the neurobiological basis of pair bonding, though this work is mostly restricted to a single model system, *Microtus* voles (Carter, 1995; Aragona and Wang, 2004; Young et al., 2011; Freeman and Young, 2013; Gobrogge and Wang, 2016). Extending this research to consider other pair bonding species, especially within early vertebrate lineages (e.g., fishes) is fundamental in understanding the generalities of prior research, but also to establish the origin and evolution of specific neurological systems that facilitate pair bonding across all vertebrate lineages (Goodson and Thompson, 2010; Goodson and Kingsbury, 2011).

1.1 An operational definition for "pair bonding"

A fundamental first step for studying pair bonding, is to unequivocally define "pair bonding". Despite extensive research on this topic, relatively few authors provide explicit definitions nor necessarily consider the specific ecological context for assessing pair bonding (Fuentes, 2000). Where definitions of pair bonding have been given, these are highly inconsistent and sometimes even contradictory (**Table 1.1**). Notably, "pair bonding" has been used in the literature to refer to a mating system (i.e., monogamous mating) (Fowler, 1995), a social system (i.e., a prolonged and pro-social affiliation between two individuals) (Fuentes, 2000; Wilson, 2000; Jolles et al., 2013), or both (Gubernick, 1994; Johnson and Burley, 1998; Quinlan and Quinlan, 2007; McGraw et al.,

2010). There are also differences in the extent to which social affiliations and/ or mating systems are defined based on selective versus exclusive intra-pair affiliation and copulation (Young and Wang, 2004), and the temporal extent of such affiliations (Lack et a. 1940).

Although mating systems and social systems (specifically, monogamy and pair bonding) often co-vary, their evolutionary basis can be quite different (Tecot et al., 2016). For example, assisted resource defense is posited to be a direct selective pressure for pair bonding, but not for monogamy (Tecot et al., 2016). Moreover, monogamous mating can occur independently of pair bonding and *vice versa* (Tecot et al., 2016). For example, red-tailed sportive lemurs, *Lepilemur reficaudatus* are monogamous but exhibit only very loose social affiliations with their sexual partners (Hilgartner et al., 2012), whereas ring-tailed lemurs (*Lemur catta*) are pair bonding, but reproductively promiscuous (Gould, 1996). Some authors implicitly confer a reproductive basis to pair bonding, by maintaining that such affiliations must involve a male and a female (e.g., Johnson and Burley, 1998; Wilson, 2000; Fowler, 1995). Pair bonding can however, occur among immature individuals (e.g., butterflyfishes; Fricke, 1986; Tricas, 1986; Pratchett et al., 2006), or individual of the same sex (e.g., Grey whales, Bagemihl, 1999; Zebra finches, Elie et al., 2011; Laysan albatrosses, Young et al., 2008; butterflyfishes, Gore, 1983; Tricas, 1986; rabbitfishes Brandl and Bellwood, 2013). Homosexual pairing occurs in a wide range of taxa (ibid), and though relatively uncommon, demonstrates that the adaptive benefits of pair bonding extend beyond reproduction.

Aside from monogamy, pair bonding is also often considered synonymous with bi-parental care (Johnson and Burley, 1998; Young and Wang, 2004), especially among birds. While species with high levels of bi-parental care (where there is extended co-operation among breeding individuals to maximize the post-hatching survival of their progeny) almost universally exhibit strong pair bonding (Kleiman, 1977; Buss, 1988, Fraley et al., 2005; McGraw et al., 2010), pair bonding and bi-parental care are nonetheless discrete and dissociable attributes (e.g., Roland and O'Connell, 2015). In mammals and birds, the incidence of bi-parental care often co-varies with monogamy and pair bonding, which is problematic in discerning the independent neural mechanisms underlying each of these behaviours (Goodson and Kingsbury, 2011; Goodson, 2013). There are however, many other vertebrate lineages that exhibit pair bonding, but little or no parental care. Most notably, there are many species of coral reef fishes that exhibit pair bonding (e.g, butterflyfishes, Fricke, 1986; Barlow, 1984, 1986) but no care for their gametes, let alone progeny, following broadcast spawning. Indeed, it is for this reason that marine fishes represent an important model system for understanding the neural basis of pair forming (discussed later).

The selective affiliation between just two individuals (pairing) is a relatively conspicuous mode of sociality, along an otherwise broad and continuous spectrum of group sizes (Tecot et al., 2016), which certainly warrants definitive recognition and

explicit scientific attention. However, pair bonding is not necessarily linked to a particular mating system, nor the need for bi-parental care (Tecot et al., 2016; Fricke, 1986; Bull et al., 1998; Pierce and Lifjeld, 1988; Roland and O'Connell, 2015). Importantly, pair bonding may be apparent at any ontogenetic stage, and occurrs between heterosexual and homosexual partners (Robertson et al., 1979; Gore, 1983; Fricke, 1986; Tricas, 1986; Bagemihl, 1999; Pratchett et al., 2006; Young et al., 2008; Elie et al., 2011; Brandl and Bellwood, 2013). While same-sex pair bonding is typically uncommon or very rare (Tricas, 1986, Pratchett et al., 2006; Young et al., 2008; Brandl and Bellwood, 2013), it also appears to be common among certain species (Robertson et al., 1979; Gore, 1983; Bagemihl, 1999). Therefore, for the purpose of this thesis, I operationally define pair bonding as *a social system characterized by a relatively enduring and pro-social affiliation between two individuals that is maintained beyond (or outside of) the process of reproduction*. This definition most closely reflects that proposed by Fuentes (2000), but is even more encompassing because it extends to non-reproductive and related individuals, and does not therefore, necessarily confer any reproductive basis to pair bonding (**Table 1.1**).

Table 3.1. Definitions or descriptions of "pair bond[ing]" put forth by relevant scientific studies. Published definitions are explicitly distinguished based on whether they consider (explicitly or implicitly) pair bonding to be a mating system, a social system, or both.

Definition or description	**Social or mating system**	**Taxa**	**Reference**
An enduring preferential association between two sexually mature adults; and is characterized by selective contact, affiliation, and copulation with the partner over a stranger (partner preference).	Both	General	Gubernick, 1994
A long-term selective social attachment between a mating pair that does not imply sexual fidelity.	Both	General	McGraw et al., 2010
A long-term affiliation between two individuals, including a sexual relationship.	Both	Humans	Quinlan and Quinlan, 2007
A social and reproductive relationship between a male and a female that share parental care duties.	Both	Birds	Johnson and Burley, 1998
A close and long-lasting association formed between a male and a female.	Social	General	Wilson, 2000

A long-term social relationship (i.e., extending beyond one breeding season), between two unrelated individuals of the opposite sex	Social	Primates	Tecot et al., 2016
A long-term association between two non-kin adults that is characterized by a set of partner-specific affiliative behaviors and energetic investment patterns.	Social	Primates	Fuentes, 2000
High levels of affiliative behavior and close proximity.	Social	Birds	Jolles et al., 2013
An extreme form of monogamy, in which one male and one female reunite for two or more successive breeding seasons.	Mating	Birds	Fowler, 1995
A selective preference for a particular mate.	Mating	General	Donaldson and Young, 2008
A heterosexual preference.	Mating	Rodents	DeVries et al., 1997

1.2 Widespread occurrence of pair bonding among vertebrates

Pair bonding is represented in every major vertebrate lineage, but is best-represented among birds (**Figure 1.1**) where 90% of species exhibit pair bonding (Lack, 1968). Pair bonding is comparatively much less common in other vertebrate lineages (**Figure 1.1**). Even among monkeys and primates, pair bonding is apparent in much less than 60% of species (Lukas and Clutton-Brock, 2013). This within-lineage rarity, but broad occurrence, suggests that pair bonding has independently evolved many times and is therefore a highly convergent phenomenon within the sub-phylum (Reichard and Boesch, 2003).

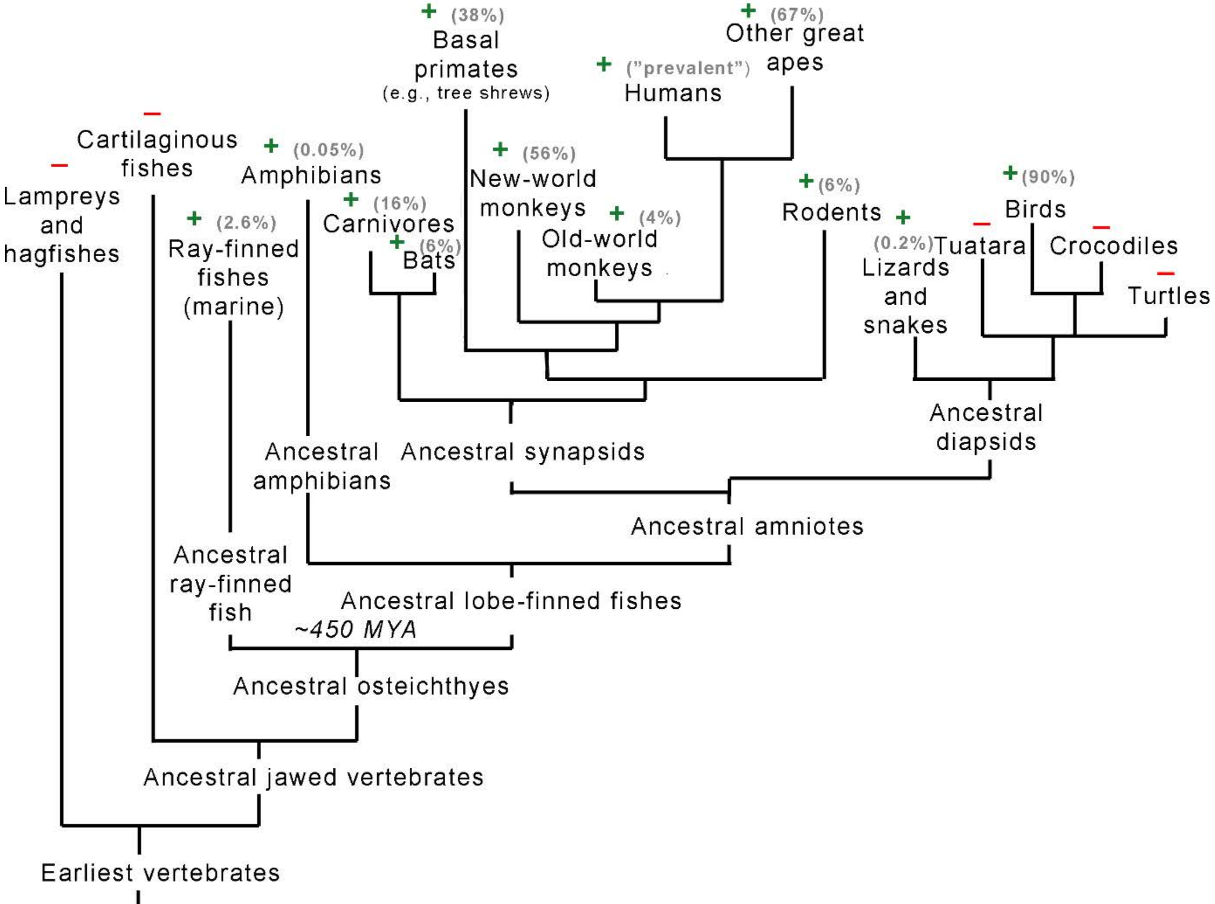


Figure 1.1. Distribution and estimated prevalence of pair bonding among select vertebrate lineages. The presence of pair bonding (+) and corresponding estimated prevalence (% spp), or absence (-) is shown for each group. Topology adopted from Butler and Hodos, 2005. *Sources for distribution of pair bonding among selected groups and estimated prevalence*: Mammals: Porton, 1983; McWilliam, 1987; Heller et al., 1993; Crooks and Van Vuren, 1996; Fuentes, 2000; Ralls et al., 2007; Munshi-South, 2008; Glenn et al., 2009; Jácomo et al., 2009; Wright et al., 2010; Marino et al., 2012; Seidler and Gese, 2012; Opie et al., 2013; Lukas and Clutton-Brock, 2013; Poessel and Gese, 2013; Jordan et al., 2014; Friesen et al., 2015; Funakoshi et al., 2015. Birds: Lack, 1968; Griffith et al., 2002; Cockburn, 2006. Reptiles: Bull, 2000; Chapple, 2003. Amphibians: Caldwell, 1997; Gillette et al., 2000; IUCN Red List, 2007; Brown et al., 2008; Brown et al., 2010. Fishes: Pratt et al., 2001; Whiteman and Côté, 2004; Froese and Pauly, 2012; Brandl and Bellwood, 2014.

For species in which it occurs, pair bonding represents a major defining feature for social structure, and has therefore garnered an extraordinary amount of attention from biologists (Goodson and Kingsbury, 2011). Scientists have especially been fascinated by pair-bonding in mammals and birds (Elmen and Oring, 1977; Dewsbury, 1988; Reichard and Boesch, 2003), perhaps because of its more direct relevance to human sociality and behavior (Reichard and Boesch, 2003; Young, 2003; Young and Wang, 2004; Freeman and Young, 2016). For these later lineages, biologists have made much progress towards achieving an integrative and complete understanding of pair bonding from a single point perspective (i.e., a perspective that addresses the trait in its current form, rather than historical events that led up to the trait) (Nesse, 2013; Bateson and Laland, 2013). This has been achieved by applying two fundamental and

complementary questions about trait biology, put forth by Tinbergen (1963): *How does it work?* (i.e., what is its proximate, mechanistic structure?), and 2) *What is it for?* (i.e., what is its potential adaptive function(s) in response to certain selection pressures?) (**Figure 1.2**). These questions, while inter-related, are logically distinct and often necessitate different scientific protocols and methods to answer (Hogan, 1994). Yet, these questions also complement each other by addressing *how* and *why* behaviors occurs, respectively (Klopfer and Hailman, 1972). In this sense, they coalesce to address the deeper, more unifying question: *How does the perception of environmental conditions trigger specific governing mechanistic processes in order to facilitate adaptive behavioral responses*? (Oliveira, 2012; O'Connell, 2013).

Substantial insight has been gained into the mechanisms underlying pair bonding among later vertebrates, especially mammals (reviewed by: Carter et al., 1995; Aragona and Wang, 2004; Young and Wang, 2004; McGraw et al., 2010; Young et al, 2011; Freeman and Young, 2013; Johnson and Young, 2015; Freeman and Young, 2016; Gobrogge and Wang, 2016). Much of this has come from studies on small rodents (f: Cricetidae, g: *Microtus*), whose tractability to laboratory settings have allowed for extensive experimental studies, which show the functional involvement of candidate neurochemical systems (Young et al, 2011; Freeman and Young, 2013; Gobrogge and Wang, 2016). Moreover, intra- and inter-specific variation in pairing behavior within the group facilitate comparative studies to infer brain region(s) in which neurochemical system may act to exert effects (Insel et al., 1994; Young et al, 2011; Freeman and Young, 2013). Collectively, these studies have helped to establish the underlying neural circuitry that is integral to pair bonding, comprising of four distinct neurochemical systems: the oxytocin, arginine vasopressin, dopamine, and opioid systems. By targeting specific functional regions of the brain, it is suggested that oxytocin and arginine vasopressin modulate aspects of social memory, while dopamine and opioids govern reward and "desire"/motivation circuits, respectively, to facilitate partner attachment (Donaldson and Young, 2016).

Various adaptive benefits are though to have either directly or indirectly promoted social monogamy among birds and mammals. i) Higher growth and survival of offspring due to bi-parental care may subsequently lead to pair bonding. ii) Increased fitness of paired individuals due to monopolization of resources (i.e., by males either defending female "resources" directly, or defending resources important to females). Finally, iii) maximizing the quality of progeny through selective mating (i.e., females may choose monogamy if males are of particularly high quality or can provide high quality resources to her and/or her offspring) may subsequently promote pair bonding. Different combinations of these adaptive benefits result in ten distinct pathways to social monogamy that are often lineage-specific (reviewed by Reichard and Boesch, 2003).

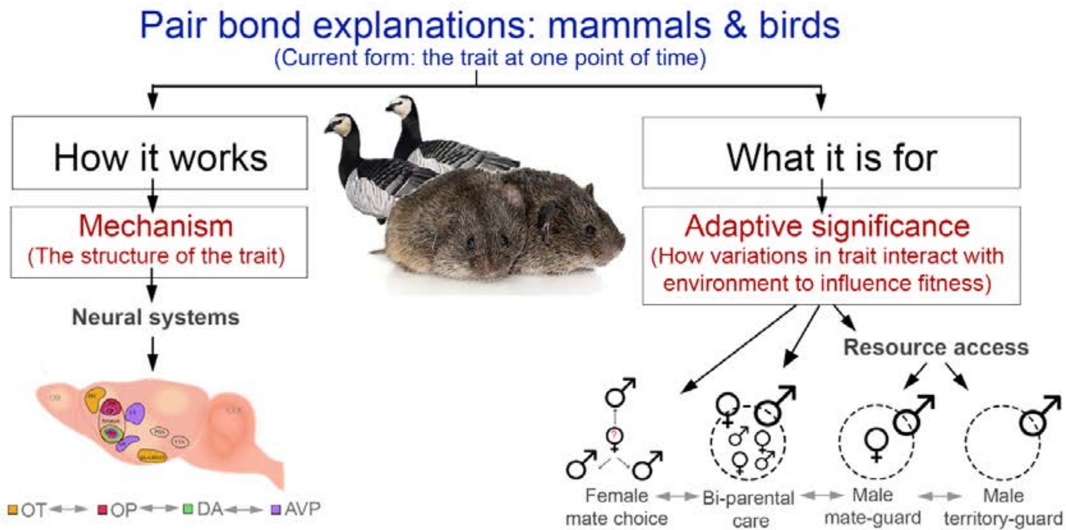


Figure 1.2. Complimentary biological explanations for how and why pair bonding occurs among later vertebrates. Explanations apply to the trait in its current form, rather than historical/ontogenetic sequences that resulted in the trait. **Mechanism (how):** Researchers have achieved a good understanding of the mechanisms that underpin pair bonding, particularly with respect to its governing neural circuitry in rodents (Young and Wang, 2004; Young, 2003; Johnson and Young, 2015; Donaldson and Young, 2016). Oxytocin (OT), arginine vasopressin (AVP), dopamine (DA) and opioids (OP) are integral (but not exclusive) systems involved (Johnson and Young, 2015; Donaldson and Young, 2016). **Adaptive significance (why):** Pair bonding primarily functions to maximize reproductive output through female mate choice, bi-parental care, and male resource guarding (Reichard and Boesch, 2003). Grey double-sided arrows indicate that neural components operate interactively, while the adaptive components can (but don't always) interact with each other, and in a lineage-specific manner. Pictures featured are classic model organisms for exploring the mechanistic basis (*Microtus ochrogaster*), and adaptive basis (*Branta leucopsis*) in mammals and birds, respectively.

1.3 Pair bonding in fishes

Compared to birds and mammals, considerably less is known about the underlying neural basis or adaptive benefits of pair bonding among reptiles, amphibians, and fishes. This is attributable, at least in part, to the limited research on pair bonding in these lineages (**Figure 1.3)**. Research into pair bonding in reptiles, amphibians, and fishes is nonetheless important. These lineages possess specific properties that facilitate a better understanding of pair bonding (Krogh's principle: Krogh, 1929), and its evolutionary history. Within all of these lineages, there exists certain closely related groups of species that display variation in pair bonding vs. non-pairing sociality, offering the opportunity for comparative research (e.g., reptiles: Whiting, 2016; amphibians: Brown et al., 2010; Roland and O'Connell, 2015; fishes: Hourigan, 1989; Dewan et al., 2011; Oldfield et al., 2013; O'Connor et al., 2016). In addition to these social properties, their use for

revealing general mechanisms of pair bonding is enriched by the recent finding that key neural substrates involved in social behaviour appear to be well conserved across vertebrates (O'Connell and Hofmann, 2011, 2012). Most broadly, because of their earlier evolutionary origins, research findings within these lineages would generate insight into the early origins of pair bonding, which when compared to existing findings among later lineages, would generate insight into the deep evolutionary history of vertebrate pair bonding—an intriguing topic among evolutionary biologists (Goodson and Kingsbury, 2011). In this sense, the following questions could begin to be addressed: *Has the repeated independent evolution of pair bonding in different vertebrate lineages been a consequence of reaching similar adaptive solutions to similar selection pressures and ecological constraints?* If so, *has this been facilitated by repeatedly co-opting similar governing neural substrates?* (Goodson and Kingsbury, 2011; O'Connell et al., 2012). The prospect for a convergent regulatory network has arisen from findings that the brain regions of vertebrates present a high degree of functional homology (Wullimann and Mueller, 2004; Broglio et al., 2005), and that socially-relevant neurochemical genes and their expression patterns across socially-relevant brain regions are both well conserved across vertebrates (O'Connell and Hofmann, 2011, 2012). Considering these pertinent reasons for exploring pair bonding in reptiles, amphibians, and fishes rapidly leads to identifying teleost (bony) fishes as the most promising and informative lineage.

Among all vertebrate lineages, teleost fishes, display the second-highest frequency of pair bonding species (second only to birds). Among the ~29,000 species (that constitute nearly half of all vertebrates) (Froese and Pauly, 2012), a least 387 species spanning 36 families in marine environments alone reportedly pair bond (data compiled from Whiteman and Côté, 2004; Brandl and Bellwood, 2014). The majority of families in which it commonly occurs occupy coral reef habitats (Whiteman and Côté, 2004; Brandl and Bellwood, 2014). In many of these families, a range of alternative social systems can also be found (e.g., in Chaetodontidae, Siganidae, Syngnathidae, Tetraodontidae, and Pomacanthidae, Brandl and Bellwood, 2014), offering several opportunities for comparative studies. Moreover, such within-family variance in sociality often occurs in a concentrated geographic region (e.g., the Indo-Pacific region), (i.e., Brandl Bellwood, 2014), controlling for this potential confound, and making observations on/ collection of wild individuals more feasible. Finally, teleosts first arose ~310 MYA (Triassic period) (Greenwood et al., 1996), though more broadly, they are extant members of ray-finned fishes (actinopterygians), whose origins date back to ~422 MYA (Silurian period) (Benton and Donoghue, 2007). Hence, teleost fishes represent

the most ancestral, earliest lineage of all key vertebrate lineages, making them best suited to generate insight into the earliest evolution of vertebrate pair bonding.

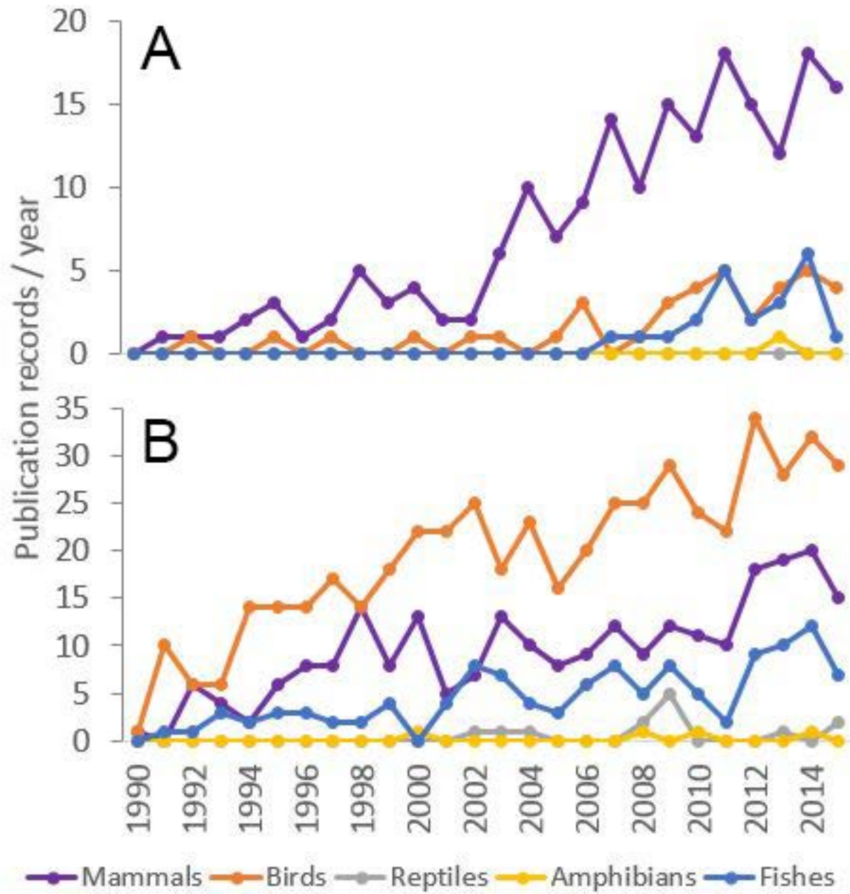


Figure 1.3. Annual number of publications on (A) the neural and (B) the adaptive basis of pair bonding in each of the five major vertebrate lineages. Data are based on the ISI Web of Science database in October, 2016. *Boolean search criteria for each lineage: Adaptive basis*: TITLE: (("pair bond*" OR monogam*) AND ([name of lineage]) OR TOPIC: (("pair bond*" OR monogam*) AND [name of lineage]). Refined by: TOPIC: ((adap* OR evolution OR origin*)). *Neural basis*: TITLE: (("pair bond*" OR monogam*) AND ([name of lineage]) OR TOPIC: (("pair bond*" OR monogam*) AND ([name of lineage]). Refined by: TOPIC: ((neuro* OR neural)). Timespan: All years. Indexes: SCI-EXPANDED, SSCI, AandHCI, CPCI-S, CPCI-SSH, ESCI, CCR-EXPANDED, IC.

Coral reef butterflyfishes (f. Chaetodontidae) appear to be foremost among teleost fishes in the prevalence of pair bonding. Of the 127 extant species of butterflyfishes, 77 are purportedly pair bonding (**Table 1.2**), accounting for 20% of all marine teleosts reported to display this sociality (data sourced from Whiteman and Côté, 2004; Brandl and Bellwood, 2014). With the exception of a few species (e.g., *C. lunulatus*), critical examination and therefore the certainty of social system is lacking for many butterflyfish species (Yabuta and Berumen, 2014). However, available data (primarily group size) suggests that they might display striking striking intra- and inter-specific variation in social systems (Reese, 1973, 1975, Hourigan, 1989; Roberts and Ormond, 1992; Yabuta and Berumen, 2014; Table 1.4); ranging from pair bonding,

aggregating, haremic, and solitary living; enabling explicit comparisons between pair bonding and non-pair bonding individuals, both within and among species (e.g., Hourigan, 1989; Roberts and Ormond, 1992; Dewan et al., 2008, 2011; Dewan and Tricas, 2011). Hourigan (1989) was among the first to exploit this apparrant interspecific variation in the sociality of coral reef butterflyfishes, revealing that pairing is associated with reliance on nutrient poor yet defendable food resources (i.e., coral), whereas gregarious schooling is associated with reliance on nutrient rich and non-defendable food resources (i.e., pelagic plankton). Additionally, territoriality is most prevalent among coral-feeding species (Roberts and Ormond, 1992). Taken together, these ecological links suggest that pairing might function for assisted defense of coral prey against competitors (Fricke, 1986; Hourigan, 1987; Roberts and Ormond, 1992).

Dewan et al. (2008) were the first to use inter-specific variation in social systems of butterflyfishes to explore the mechanistic basis of pairing. They found that a monogamous, pairing, and territorial species (*Chaetodon multicinctus*) had larger arginine vasotocin immune-receptive (AVT-ir) neurons in the pre-optic brain area (POA) and greater AVT-ir neuron fibre densities in several brain regions than a shoaling, non-territorial species (*C. miliaris*). A subsequent, broader comparison of seven species revealed that AVT-ir varicosity density in the ventral portion of the ventral telencephalon (Vv) was related to species-typical agonism, mating system, and social group size (Dewan et al., 2011). Importantly, there is scope for such comparative analysis to be designed in a highly controlled manner in these organisms, since several aspects of their ecology (Cole and Pratchett, 2014; Pratchett, 2014; Yabuta and Berumen, 2014) biogeography (Kulbicki et al., 2014), and phylogeny (Fessler and Westneat, 2007; Bellwood et al., 2010; Cowman and Bellwood, 2011) are becoming firmly established. Finally, it is conceivable that comparatively derived correlates can be tested for causality in these organisms, owing to their tractability to both *in situ* (e.g., Fricke, 1986; Hourigan, 1987) and laboratory (e.g., Dewan and Tricas, 2011) experimentation.

Table 1.4. Tentative species-typical social systems in butterflyfishes, based on predominant group size. Also shown are species' mating systems.

Genus/ minor clade	Species	Predominant group size (%)	Mating system	Reference
Amphichaetodon	*howensis*	Pair		Kuiter, 2002 (QL, NPR)
Amphichaetodon	*melbae*			
Chaetodon (Clade 1)	*hoefleri*	Pair		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 1)	*marleyi*			
Chaetodon (Clade 1)	*robustus*	Pair		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 2)	*argentatus*	Pair-Group		Kuiter, 2002

Chaetodon (Clade 2)	*assarius*	Group		Allen et al., 1998 (QL, NPR)
Chaetodon (Clade 2)	*blackburnii*			
Chaetodon (Clade 2)	*burgessi*	Pair		Allen et al., 1998 (QL, NPR)
Chaetodon (Clade 2)	*citrinellus*	Pair (85.6%)		Reese, 1975 (QN, PR); Yabuta, 2007 (QN, PR); Yabuta and Berumen, 2014 (QN, NPR);
Chaetodon (Clade 2)	*daedalma*	School		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 2)	*declivis*			
Chaetodon (Clade 2)	*dialeucos*	Pair		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 2)	*dolosus*			
Chaetodon (Clade 2)	*excelsa*			
Chaetodon (Clade 2)	*flavocoronatus*			
Chaetodon (Clade 2)	*fremblii*	Solitary	Polygynous	Yabuta and Berumen, 2014 (QL, NPR)
Chaetodon (Clade 2)	*guentheri*	School		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 2)	*guttatissimus*	Pair (92%)		M. Pratchett, Unpub Data – Chagos (QN, NPR)
Chaetodon (Clade 2)	*guyotensis*	Pair		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 2)	*interruptus*	Pair (77%)		M. Pratchett, Unpub Data – Chagos (QN, NPR)
Chaetodon (Clade 2)	*jayakari*			
Chaetodon (Clade 2)	*kleinii*	Pair (80%)		Yabuta, 2007 (QN, PR)
Chaetodon (Clade 2)	*litus*	School		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 2)	*madagaskariensis*	Pair (100%)		M. Pratchett, Unpub Data – Chagos (QN, NPR)
Chaetodon (Clade 2)	*mertensii*	Pair		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 2)	*miliaris*	Solitary		Yabuta and Berumen, 2014 (QL, NPR)
Chaetodon (Clade 2)	*mitratus*			
Chaetodon (Clade 2)	*modestus*	Group		Allen et al., 1998 (QL, NPR)
Chaetodon (Clade 2)	*multicinctus*	Pair (83.3%)	Monogamous	Reese, 1975 (QN, PR); Yabuta, 2007 (QN, PR); Yabuta and Berumen, 2014 (QN, NPR)
Chaetodon (Clade 2)	*nippon*	Group		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 2)	*paucifasciatus*	Pair (70%)	Monogamous	Yabuta, 2007 (QN, PR)
Chaetodon (Clade 2)	*pelewensis*	Pair (73.3%)		Bouchon-Navaro, 1981 (QN, PR); Yabuta and Berumen, 2014 (QN, NPR)

Chaetodon (Clade 2)	*punctatofasciatus*	Pair (73.3%)		Reese, 1975 (QN, PR); Yabuta and Berumen, 2014 (QN, NPR)
Chaetodon (Clade 2)	*quadrimaculatus*	Pair (80%)	Monogamous	Reese, 1975 (QN, PR); Bouchon-Naravo, 1981 (QN, PR); Yabuta, 2007 (QN, PR); Yabuta and Berumen, 2014 (QN, NPR)
Chaetodon (Clade 2)	*sanctaehelenae*	Pair		Allen et al., 1998 (QL, NPR)
Chaetodon (Clade 2)	*sedentarius*	Pair		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 2)	*smithi*	School		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 2)	*tinkeri*	Pair-Group		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 2)	*trichrous*	Group		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 2)	*unimaculatus*	Pair (72.2%)		Reese, 1975 (QN, PR); Bouchon-Navaro, 1981 (QN, PR); Yabuta, 2007 (QN, PR); Yabuta and Berumen, 2014 (QN, NPR)
Chaetodon (Clade 2)	*xanthurus*	Pair		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 3)	*andamanensis*	Pair-Group		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 3)	*aureofasciatus*	Solitary (84.4%)		Reese, 1975 (QN, PR); Yabuta and Berumen, 2014 (QN, NPR)
Chaetodon (Clade 3)	*austriacus*	Pair (82.2%)		Fricke, 1986 (QN, PR),Yabuta and Berumen, 2014 (QN, NPR)
Chaetodon (Clade 3)	*baronessa*	Pair (55.6%)		Yabutta, 2007 (QN, PR)
Chaetodon (Clade 3)	*bennetti*	Solitary (86.1%)		Bouchon-Navaro, 1981 (QN, PR); Yabuta, 2007 (QN, PR); Yabuta and Berumen, 2014 (QN, NPR)
Chaetodon (Clade 3)	*larvatus*	Pair		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 3)	*lunulatus*	Pair (95%)	Monogamous	Reese, 1975 (QN, PR); Bouchon-Naravo, 1981 (QT, PR); Yabuta, 2007 (QN, PR); Yabuta and Berumen, 2014 (QN, NPR)
Chaetodon (Clade 3)	*melapterus*	Pair		Kuiter, 2002 (QL, NPR)

Chaetodon (Clade 3)	*meyeri*	Pair (74%)		M. Pratchett, Unpub Data – Chagos (QN, NPR)
Chaetodon (Clade 3)	*octofasciatus*	Group		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 3)	*ornatissimus*	Pair (58.9%)	Monogamous	Reese, 1975 (QN, PR)
Chaetodon (Clade 3)	*plebeius*	Solitary (82.2%)		Reese, 1975 (QN, PR); Yabuta, 2007 (QN, PR); Yabuta and Berumen, 2014 (QN, NPR)
Chaetodon (Clade 3)	*rainfordi*	Solitary (76.7%)		Reese, 1975 (QN, PR)
Chaetodon (Clade 3)	*reticulatus*	Pair (58.3%)		Reese, 1975 (QN, PR); Bouchon-Navaro, 1981 (QN, PR); Yabuta and Berumen, 2014 (QN, NPR)
Chaetodon (Clade 3)	*speculum*	Solitary (72.2%)		Reese, 1975 (QN, PR); Yabuta, 2007 (QN, PR); Yabuta and Berumen, 2014 (QN, NPR)
Chaetodon (Clade 3)	*triangulum*	Pair (70%)		Reese, 1975 (QN, PR)
Chaetodon (Clade 3)	*tricinctus*			
Chaetodon (Clade 3)	*trifascialis*	Solitary (93.3%)	Polygynous (Haeremic)	Bouchon-Navaro, 1981 (QN, PR); Fricke, 1986 (QN, PR); Yabuta and Berumen, 2014 (QN, NPR)
Chaetodon (Clade 3)	*trifasciatus*	Pair (96%)		M. Pratchett, Unpub Data – Chagos (QN, NPR)
Chaetodon (Clade 3)	*zanzibariensis*	Pair (60%)		M. Pratchett, Unpub Data – Chagos (QN, NPR)
Chaetodon (Clade 4)	*adiergastos*	School		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 4)	*auriga*	Pair (63.3%)		Reese, 1975 (QN, PR); Bouchon-Navaro, 1981 (QN, PR); Fricke, 1986 (QN, PR); Yabuta, 2007 (QN, PR); Yabuta and Berumen, 2014 (QN, NPR)
Chaetodon (Clade 4)	*auripes*	Pair (82.2%)		Yabuta, 2007 (QN, PR)
Chaetodon (Clade 4)	*capistratus*	Pair (75%)	Monogamous	Yabuta, 2007 (QN, PR)
Chaetodon (Clade 4)	*collare*	Pair		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 4)	*decussatus*	Pair		Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 4)	*ephippium*	Pair (66.7%)		Reese, 1975 (QN, PR); Bouchon-Navaro,

			1981 (QN, PR); Yabuta, 2007 (QN, PR); Yabuta and Berumen, 2014 (QN, NPR)
Chaetodon (Clade 4)	*falcula*	Pair (92%)	M. Pratchett, Unpub Data – Chagos (QL, NPR)
Chaetodon (Clade 4)	*fasciatus*	Pair (40%)	Yabuta and Berumen, 2014 (QN, NPR)
Chaetodon (Clade 4)	*flavirostris*	Pair	Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 4)	*gardineri*	Pair-Group	Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 4)	*humeralis*	Pair-Group	Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 4)	*leucopleura*	Solitary	Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 4)	*lineolatus*	Solitary (47%)	Reese, 1975 (QN, PR); Fricke, 1986 (QN, PR); Yabuta, 2007 (QN, PR); Yabuta and Berumen, 2014 (QN, NPR)
Chaetodon (Clade 4)	*lunula*	Pair (39%)	Reese, 1975 (QN, PR); Bouchon-Navaro, 1981 (QN, PR); Yabuta, 2007 (QN, PR); Yabuta and Berumen, 2014 (QN, NPR)
Chaetodon (Clade 4)	*melannotus*	Solitary (86.7%)	Yabuta, 2007 (QN, PR)
Chaetodon (Clade 4)	*mesoleucos*	Pair	Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 4)	*nigropunctus*	Pair (80%)	M. Pratchett, Unpub Data – Oman (QN, NPR)
Chaetodon (Clade 4)	*ocellatus*	Pair	Allen et al., 1998 (QL, NPR)
Chaetodon (Clade 4)	*ocellicaudus*	Pair	Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 4)	*oxycephalus*	Pair	Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 4)	*pictus*	Pair	Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 4)	*rafflesi*	Pair (84.4%)	Yabuta, 2007 (QN, PR)
Chaetodon (Clade 4)	*selene*	Pair	Kuiter, 2002 (QL, NPR)
Chaetodon (Clade 4)	*semeion*	Pair	Allen et al., 1998 (QL, NPR)
Chaetodon (Clade 4)	*semilarvatus*	Pair (80%)	Fricke, 1986 (QL, PR)
Chaetodon (Clade 4)	*striatus*	Pair	Menez et al., 2003 (QL, NPR)
Chaetodon (Clade 4)	*ulietensis*	Pair (57.2%)	Bouchon-Navaro, 1981 (QN, PR)
Chaetodon (Clade 4)	*vagabundus*	Pair (74.4%)	Reese, 1975 (QN, PR); Bouchon-Navaro, 1981 (QN, PR);

				Yabuta, 2007 (QN, PR); Yabuta and Berumen, 2014 (QN, NPR)
Chaetodon (Clade 4)	*wiebeli*	Solitary		Allen et al., 1998 (QL, NPR)
Chaetodon (Clade 4)	*xanthocephalus*	Solitary		Allen et al., 1998 (QL, NPR)
Chelmon	*marginalis*	Pair		Kuiter, 2002 (QL, NPR)
Chelmon	*muelleri*			
Chelmon	*rostratus*	Pair		Kuiter, 2002 (QL, NPR)
Chelmonops	*curiosus*	Pair		Kuiter, 2002 (QL, NPR)
Chelmonops	*truncatus*	Pair		Kuiter, 2002 (QL, NPR)
Coradion	*altivelis*	Pair		Kuiter, 2002 (QL, NPR)
Coradion	*chrysozonus*	Pair		Kuiter, 2002 (QL, NPR)
Coradion	*melanopus*	Pair		Kuiter, 2002 (QL, NPR)
Forcipiger	*flavissimus*	Pair		Kuiter, 2002 (QL, NPR)
Forcipiger	*longirostris*	Pair		Kuiter, 2002 (QL, NPR)
Hemitaurichthys	*multispinosus*			
Hemitaurichthys	*polylepis*	School		Kuiter, 2002 (QL, NPR)
Hemitaurichthys	*thompsoni*			
Hemitaurichthys	*zoster*	School		Kuiter, 2002 (QL, NPR)
Heniochus	*acuminatus*	Pair-Group		Kuiter, 2002 (QL, NPR)
Heniochus	*chrysostomus*	Pair		Kuiter, 2002 (QL, NPR)
Heniochus	*diphreutes*	School		Kuiter, 2002 (QL, NPR)
Heniochus	*intermedius*	Pair (76%)		Fricke, 1986 (QN, PR)
Heniochus	*monoceros*	Pair		Kuiter, 2002 (QL, NPR)
Heniochus	*pleurotaenia*	Pair/School		Kuiter, 2002 (QL, NPR)
Heniochus	*singularis*	Pair		Kuiter, 2002 (QL, NPR)
Heniochus	*varius*	Pair		Kuiter, 2002 (QL, NPR)
Johnrandallia	*nigrirostris*	School		Kuiter, 2002 (QL, NPR)
Prognathodes	*aculeatus*	Solitary (91.1%)	Polygynous	Yabuta and Berumen, 2014 (QN, NPR)
Prognathodes	*aya*			
Prognathodes	*basabei*	Trio		Pyle and Kosaki 2016 (QL, NPR)
Prognathodes	*brasiliensis*			
Prognathodes	*dichrous*	Pair		Kuiter, 2002 (QL, NPR)
Prognathodes	*falcifer*			
Prognathodes	*guezei*			
Prognathodes	*guyanensis*			
Prognathodes	*guyotensis*			
Prognathodes	*marcellae*			
Prognathodes	*obliquus*			

Notes: QL = data is qualitative (descriptive observations reported by observer); QN = data is quantitative (based on numerical comparisons and/or statistical inferences). NPR = data from non-peer reviewed source (i.e., pers. communication, observations, books, or dissertation); PR = data from peer-reviewed source (i.e., peer-reviewed scientific journal).

1.4 Thesis aim and outline

The overall aim of my thesis was to investigate the i) underlying neurobiological basis and ii) ecological benefits of pair bonding in fishes, and specifically, coral reef butterflyfishes. The foremost step in achieving these aims, was to test the utility of coral butterflyfishes for highly controlled intra- and inter-species comparative and experimental neural research (**Chapter 2)**, thereby further establishing butterflyfishes as a model system for neurobiological research (see Dewan et al., 2011). This represents one of the first marine fish model system for examining the neurobiology of prosocial relationships (see Dewan et al., 2011), which is important both to understand the origin and evolution of different social systems, but also to explore the neural substrates of social attachment independently of parental care. In **Chapter 3**, I then use the butterflyfish model system for conducting integrative (comparative and experimental) research to establish the underlying neurochemical and anatomical basis of pair forming in teleost fishes. This novel research provides the first working neural circuitry model for teleost pair bonding, from which specific hypotheses can be tested in the future. When compared to that of mammals, it furthermore generates insight into the evolutionary history of vertebrate pair bonding regulatory mechanisms. Finally, in **Chapter 4**, I conduct *in situ* comparative and experimental studies on two species (*C. lunulatus* and *C. baronessa*) to test the hypothesis that pairing may function for cooperative resource defence purposes, and that partner fidelity contributes to this function, thereby providing insight into the ecological significance of partner formation and fidelity in these species.

This thesis represents one of the few, but growing number of studies that answers Tinbergen's call for integrating physiology and ecology in order to fundamentally address *how* and *why* a trait occurs within a single organism—leading to a deeper, more holistic understanding of its biological significance (e.g., Bateson and Laland, 2013, bird song). By using this approach to address pair bonding in fishes, this thesis ultimately sheds new light onto whether the convergence of pair bonding across vertebrates is a consequence of organisms reaching similar ecological solutions to environmental challenges through the repeated co-option of similar regulatory neural systems. In this light, we'll be able to more accurately assess the uniqueness of proximal reasons for *how*, and ecological reasons for *why* pair bonding exists in our own species, relative to non-human animals.

Aside from my PhD research (described above) I was also involved in several additional studies of the ecology and behaviour in coral reef fishes during my candidature. In the first of these projects, we explored effects of coral bleaching on the physiological condition of *Dascyllus aruanus*, an obligate coral-dwelling damselfish. Climate induced coral-bleaching is one of the most significant threats to coral reef ecosystems (Hoegh-Guldberg et al. 2007), and severe and widespread incidences of coral bleaching often results in significant declines in the abundance of many reef fishes,

especially those that rely on corals for food or habitat (Pratchett et al. 2008). However, the sub-lethal effects of coral bleaching on coral habitat dependent fishes are poorly understood. Interestingly, this study (Coker et al. 2015 – Appendix B) showed that prolonged association with bleached coral hosts had no effect on the body condition for *D. aruanus*. This study suggests that there are no ecological constraints for non-corallivorous fishes to associate with bleached corals, though they may be exposed to higher rates of predation (Coker et al. 2009). In a separate study, we also tested whether there might be indirect ecological benefits of high coral cover for the non-corallivorous butterflyfish, *Chaetodon auriga*. *Chaetodon auriga* has no specific reliance on live corals for either food, shelter or recruitment, but often exhibits declines in abundance following extensive coral loss (e.g., Bouchon-Navaro et al. 1985). Accordingly, we explored variation in feeding behavior of *C. auriga* among sites with varying levels of live coral cover, showing that feeding rates were significantly and positively correlated with live coral cover (Pratchett et al. 2015 – Appendix B). This study suggests that *C. auriga* may be negatively affected by localized coral depletion because its prey is more abundant in coral-rich habitats.

Chapter 2: Butterflyfishes (f: Chaetodontidae) as a model system for investigating pair bonding in fishes

2.1 Abstract

Select model species (mostly mammal and bird *spp*) have informed the neural and ecological basis of vertebrate pair bonding. Pair bonding is common within certain families of fishes (e.g., butterflyfishes), but neural mechanisms remain largely unknown. Studying pair bonding in fishes is important, in order to understand the early origins of this social system. Moreover, pair bonding fishes (unlike mammals and birds) often lack parental care, allowing neural substrates of social attachment to be distinguished from those of parental attachment. Pairing sociality is suggested to vary within and among closely related species of butterflyfishes (f: Chaetodontidae), enabling comparative approaches towards identifying neural correlates of pairing. However, because butterflyfish sociality lacks critical analysis, it remains uncertain. Moreover, tests of butterflyfish pair bonding behavior in the laboratory would enable experimental examination of pair bonding; however, remain absent. This study first critically analyzed the sociality of six species of wild butterflyfish, establishing their utility for comparatively and experimentally researching pair bonding. Based on available data, I hypothesized that among six closely related species, three (*Chaetodon baronessa, C. lunulatus,* and *C. vagabundus*) would be predominantly pair bonding, and three (*C. rainfordi, C. plebeius,* and *C. trifascialis*) would be predominantly solitary. Specifically, I tested the predictions that the former three species would predominantly occur in heterosexual, enduring pairs that exhibited selective affiliation towards partners, and selective agonism towards non-partners; whereas the latter three would predominantly occur in solitude, and exhibit little, non-selective affiliation with another individual. Field observations, conducted at Lizard Island in the northern Great Barrier Reef, revealed clear inter-specific differences in sociality as predicted, supporting my initial hypothesis. Moreover, even for species, such as *C. lunulatus* that are predominantly pair bonding, a significant proportion of adult individuals occur as solitary individuals. Secondly, I tested the hypothesis that pair bonding *C. lunulatus* males would display preferential affiliation with partners in captivity. Males placed in three-chamber experimental aquaria display strong preferential affiliation with their established female partner over a non-partner female conspecific. Collectively, these results show that these butterflyfishes are tractable models for comparative and experimentally studying teleost pair bonding.

2.2 Introduction

Pair bonding is often ascribed a reproductive basis, but may also arise due to other adaptive benefits, such as increased efficiency of territorial defence. Pair bonding has independently evolved many times across vertebrate lineages (Reichard and Boesch, 2003) and there exists a rich body of literature on the adaptive basis of this social system (reviewed by: Wilson, 2000; Elmen and Oring, 1977; Wittenberger and Tilson, 1980; Bull, 2000; Reichard and Boesch, 2003; Whiteman and Côté, 2004; Lukas and Clutton-Brock, 2013; Opie et al., 2013). Research on pair forming species is now increasingly focussed on establishing the proximal, neural basis of pair bonding, largely due to its implications for the neural mechanisms of human pro-sociality (Carter, 1998; Young and Wang, 2004; Young, 2003; Young, 2009), anti-social psychological disorders (Aragona and Wang, 2004; Volkmar, 2001), and physical health (Young and Wang, 2004). To date, this research has been largely conducted with well-established mammalian model species/systems, and especially *Microtus* voles (reviewed in: Carter et al., 1995; Aragona and Wang, 2004; Young and Wang, 2004; McGraw and Young, 2010; McGraw et al., 2010; Young et al, 2011; Freeman and Young, 2013; Johnson and Young, 2015; Gobrogge and Wang, 2016). However, there is strong impetus for broadening the range of model systems used to explore the neural basis of pair bonding to earlier lineages (i.e., to reptiles, amphibians, and fishes), particularly for understanding the early origin and subsequent evolution of neural circuitries of pair bonding.

In situ behavioral observations on wild populations are a critical first step towards establishing the existence and diversity of pair bonding within and among species (Reese, 1975; Getz and Hofmann, 1986; Caldwell, 1997). Populations that exhibit strong variation in pairing sociality between individuals or closely related species are particularly useful for comparatively assessing both the adaptive (Hourigan, 1989; Ribble, 2003; Brown et al., 2010) and the mechanistic (Phelps et al., 2010; Ondrasek, 2016) bases of pair bonding. For example, intra-specific comparative assays in the prairie vole, *Microtus ochrogaster*, have highlighted a role for the nonapeptides arginine vasopressin and oxytocin in modulating pair bonding in mammals (Ophir et al., 2008, 2012; Mabry et al., 2011; Zheng et al., 2013; Okhovat et al., 2015). Such comparisons are especially informative when conducted on wild populations, since lab-reared colonies can constrain natural genotypic and phenotypic variation over time (Phelps et al., 2010; Ondrasek, 2016). Inter-species comparisons; when controlling for potential confounds such as phylogenetic independence, life history, and behavioral ecology (Krebs, 1990; Harvey and Pagel, 1991); can illuminate general principals that may not be apparent in a single species (Hofmann et al., 2014; Taborsky et al., 2015; Weitekamp and Hofmann, 2017). However, with the exception of voles, systems for comparatively studying pair bonding have been established for few species (but see teleosts: Oldfield et al., 2013; O'Connor et al., 2015, 2016; and birds: Adkins-Regan, 2016).

Beyond comparative physiological studies, functional (e.g., pharmacological) experiments provide the ultimate insights into the neurochemical basis of pair forming, as well as other social affiliations and behaviours. Functional experiments often involve the administration of a neurochemical (e.g., arginine vasopressin (AVP)) or a corresponding antagonist (or blockade), combined with explicit tests of social behaviour to clearly establish the role of specific neurochemicals in moderating the expression of certain behavioural phenotypes. Parker and Lee (2001) for example, showed that administration of AVP initiates parental care in a non-monogamous species of meadow vole, *M. pennsylvanicus*. The two limiting factors for effective functional experiments are i) the development of effective neurochemical antagonists (Manning et al., 2008), and ii) appropriate testing apparatus to quantify specific changes in behaviour (Young et al., 2011). Changes in pair bonding are typically measured based on "two-choice" proximity (or partner preference) assays (Williams et al., 1992; Adkins-Regan, 1998, 2016), whereby the focal individual (typically a male) is placed between two different potential partners, which themselves cannot interact. Partner preference is then measured based on the proportion of time that the focal individual affiliates with one partner over another (Williams et al., 1992; Adkins-Regan 1998, 2016; Young et al., 2011), often based on their relative proximity. In addition, positive or affiliative (e.g., huddling and singing) and negative or agonistic (e.g., growling and lunging) behaviours towards either of the potential partners are often quantified to further resolve partner preferences (Williams et al., 1992; Young et al., 2011; Adkins-Regan, 2016). Partner preference assays are commonly used in pharmacological studies with birds and mammals, but could also be adapted for other earlier vertebrates (or amniotes), such as fishes.

Relatively little is known about the neurobiology of pair bonding in earlier vertebrates (Oldfield and Hofmann, 2011; O'Connell and et al., 2012; O'Connor et al., 2016), but this is important in understanding the origin and evolution of specific neurological systems that facilitate pair bonding. Importantly, pair bonding is prevalent among earlier vertebrates, even though certain neurochemical systems (e.g., AVP and oxytocin systems) that are important in promoting pair bonding among mammals are not present in earlier vertebrates. There are however, ancient homologs of these systems (see Chapter 3), which may have been fundamental in enabling independent evolution of pair bonding across all vertebrate lineages (O'Connell and Hofmann, 2011, 2012). Earlier vertebrates (especially fishes) also provide a significant advantage over birds and mammals in understanding the neural underpinning of pair bonding, because they, in some cases, lack any parental care. A recognizable limitation of established model systems (e.g., mammal system: *Microtus* voles; bird system: *Coturnix* quails) is that in one system or another, species differences in pairing behavior co-vary with differences in several other behavioural or ecological attributes, including parental care, general social affiliation, and territoriality (Goodson et al., 2006; Adkins-Regan, 2016; Gutierrez and Domjan, 1996; Domjan and Hall, 1986; Oldfield et al., 2013; O'Connor et

al., 2015, 2016). Since shared neuroendocrine mechanisms have been shown to regulate all of these attributes (Goodson and Kingsbury, 2011; Oldfield et al., 2015), it is difficult to use these systems to isolate specific neural mechanisms responsible for pair bonding from those of these other attributes, which are common to the majority of pair forming species (especially bi-parental care) (Goodson and Kingsbury, 2011).

Pair bonding is prevalent within certain families of teleosts (Barlow, 1984; Whiteman and Côté, 2004; Brandl and Bellwood, 2014) and is especially those inhabiting coral reef habitats. Approximately 17% of coral reef fishes across 29 families from the Indo-Pacific are reported to form pairs (Brandl and Bellwood, 2014). More than 50% of these pair bonding species belong to just five families, including Malacanthidae (where 100% of species pair bond), Chaetodontidae (83%), Siganidae (60%), Syngnathidae (56%), and Ptereleotridae (56%) (Brandl and Bellwood, 2014). Despite the preponderance of pair bonding in tropical marine fishes, the neurobiology of pair bonding in these organisms has rarely been studied (but see Dewan et al. 2008, 2011). A model system is, however, becoming established for freshwater cichlids, whose inter-specific variation in sociality, established phylogeny, and amiability to captive care, provide considerable opportunities for both comparative (Oldfield et al., 2013; O'Connor et al., 2016) and functional tests of pair bonding (Oldfield and Hofmann, 2011; O'Connell and Hofmann, 2012).

Butterflyfishes (f: Chaetodontidae) appear to be ideal model organisms for both comparative and experimental research on pair bonding in marine fishes. Among the 127 extant species of butterflyfishes (Bellwood and Pratchett 2014), 77 are reported to form pairs (**Table 1.2**). Although the sociality of most butterflyfishes is yet to be assessed critically (Yabuta and Berumen, 2014), available data (mostly species-typical group size) suggests that butterflyfishes also display spectacular diversity in sociality, both within (Reese, 1975; Yabuta, 2007) and among species (Reese, 1975; Yabuta, 2007; Yabuta and Berumen, 2014). Dewan et al. (2008) were the first to exploit such natural variation for mechanistic research. They found that a monogamous, pairing, and territorial species (*Chaetodon multicinctus*) had larger arginine vasotocin immune-receptive (AVT-ir) neurons in the pre-optic brain area (POA) and greater AVT-ir neuron fiber densities in several brain regions than a closely related shoaling, non-territorial butterflyfish species (*C. miliaris*). A subsequent, broader comparison of seven species in four phylogenetic clades revealed that AVT-ir varicosity density in the ventral portion of the ventral telencephalon (Vv) was related to species-typical agonism, mating system, and social group size (Dewan et al., 2011). There is good scope for intra- and inter-species comparisons of pair bonding vs. non-pair bonding to be made in a highly controlled manner in these organisms, since several aspects of their ecology (Cole and Pratchett, 2014; Pratchett, 2014; Yabuta and Berumen, 2014), biogeography (Kulbicki et al., 2014), and phylogeny (Fessler and Westneat, 2007; Bellwood et al., 2010; Cowman and Bellwood, 2011) are well studied. A notable feature in this respect is that regardless of

social system, butterflyfishes are broadcast spawners that effectively display no parental care (Fricke, 1986; Roberts and Ormond, 1992), and would therefore provide the first and foremost insights into the neural basis of pair bonding independent of this potential confound.

The purpose of this study was to establish the utility of coral reef butterflyfishes as a new model system for studying pair bonding, amenable to both comparative and functional analyses, wherein the effects of pairing are completely independent of parental care. Specifically, I sought to conduct *in situ* field studies to assess intra- and inter-specific variation in sociality (i.e., pair bonding vs. non-paired) among sympatric butterflyfishes. To characterize the sociality of sympatric butterflyfishes, I focused on features that are routinely recognized as characteristic of pair bonding across taxa, that are useful for distinguishing pairing from non-pairing phenotypes, and that are likely to be ecologically relevant to butterflyfishes. These features include i) the predominance of group sizes of two individuals (Gubernick, 1994; Fuentes, 2000; Quinlan and Quinlan, 2007; Yabuta, 2007; Gregson et al., 2008), ii) selective affiliation with a distinct partner (Gubernick, 1994; Fuentes, 2000; McGraw and Young, 2010), which in the case of fishes, may be expressed as proximate and parallel (i.e., "pair") swimming (Fricke, 1986; Hourigan, 1989), iii) selective agonism towards non-partners (Yabuta, 2000; Young et al., 2011; Adkins-Regan, 2016), iv) heterosexual pair composition (Reese, 1975; Fricke, 1986; Hourigan, 1989; DeVries et al., 1997; Wilson, 2000; Pratchett, 2006), and v) long-term partner fidelity/endurance (Reese, 1973; Fricke, 1986; Tricas, 1986; Driscoll and Driscoll, 1988; Gubernick, 1994; Yabuta, 1997, 2000; Fuentes, 2000; Wilson, 2000; Quinlan and Quinlan, 2007; McGraw and Young, 2010). Based on available data, I hypothesized that among six closely related species, three (*Chaetodon baronessa, C. lunulatus,* and *C. vagabundus*) would be predominantly pair bonding, and three (*C. rainfordi, C. plebeius,* and *C. trifascialis*) would be predominantly solitary. Specifically, I tested the predictions that the former three species would predominantly occur in heterosexual, enduring pairs that exhibited selective affiliation towards partners, and selective agonism towards non-partners; whereas the latter three would predominantly occur in solitude, and exhibit little, non-selective affiliation with another individual. Secondly, I sought to develop a partner preference assay appropriate for coral reef butterflyfishes, which would enable experimental test of pair bonding (see Chapter 3). Specifically, I tested the hypothesis that pair bonding *C. lunulatus* males would display preferential affiliation towards partners over non-partner female conspecifics in captivity.

2.3 Methods

2.3.1 Study sites and species

This research was conducted at Lizard Island, located in northern section of Australia's Great Barrier Reef (14°40'S, 145°27'E), where there is a high diversity of butterflyfishes (mostly *Chaetodon* spp.) living in sympatry (Pratchett, 2005). Field surveys were conducted on the north-western side of Lizard Island, where there are numerous distinct platform reefs that are easily accessible. A total of six species (*Chaetodon lunulatus, C. baronessa, C. vagabundus, C. plebeius, C. rainfordi,* and *C. trifascialis*) were considered during this study, which co-occur within shallow reef habitats (Pratchett, 2005) and are closely related congeners (Bellwood et al., 2010; Cowman and Bellwood, 2011). Prior research conducted at Lizard Island provides important information on the feeding ecology (Berumen et al., 2005; Pratchett, 2005; Lawton et al., 2012; Pratchett et al., 2015), demography (Berumen, 2005), and habitat associations (Berumen et al., 2005) of locally abundant *Chaetodon* species. There has not, however, been any research into the sociality of these butterflyfishes at this location.

2.3.2 Inter- and intra-specific variation in sociality for Chaetodon *butterflyfishes*

2.3.2.1 Characterizing sociality based on group size

In situ field studies to establish the sociality of butterflyfishes were conducted in winter (April-July) in 2013 and 2015, to intentionally avoid summer periods where spawning is expected to predominantly occur (but see Ralston 1981). Moreover, only individuals that were within 80% of the asymptotic size for the species, and therefore likely to be reproductively mature (Pratchett et al., 2006) were considered in this study. Sociality was assessed by quantifying the frequency distribution of group size for each of the six study species. Field *s*urveys were conducted from 0800 to 1800 and along $200m^2$ (50m x 4m) belt transects. Six replicate transects were run at each of five randomly selected platform reefs. During surveys, each individual (or group of individuals) from the focal species within the transect area was followed for a 5-min observation period. Group size was recorded for each focal species and determined by the number of individuals (either 1, 2, or 3+ individuals) that displayed proximate swimming (within 1.5m distance) for at least 3 consecutive minutes during a 5-minute observation period. Sample sizes varied in accordance with variation in abundance among the six study species: *C. rainfordi* (n=48), *C. plebeius* (n=61), *C. baronessa* (n=76), *C. lunulatus* (n=98), *C. trifascialis* (n=43), *C. vagabundus* (n=55). To explicitly test whether group sizes were statistically non-random within each species, frequency analyses were based on the number of groupings (not the number of individuals in each group), and analysed using a chi-squared homogeneity test in R (R Core Team, 2014).

2.3.2.2 Socially characterizing paired vs. solitary group sizes

To further validate sociality of the six focal species (*C. baronessa, C. lunulatus, C. plebeius, C. rainfordi C. trifascialis*, and *C. vagabundus*), field observations were conducted to specifically characterize social affiliation and agonism among group members (e.g., partners) versus conspecifics that were non-group members. *In situ* behavioural observations were conducted on snorkel at randomized times throughout the day across five randomly selected reefs. Focal individual(s) within the group were identified, and then observed from a distance of 2-5 metres. Focal individuals were allowed three minutes to acclimate to observers' presence. For predominantly paired species, proximate and parallel swimming (see **Figure 2.1** for schematic of parallel swimming) with partner relative to non-partner were recorded; and for predominantly solitary species and *C. lunulatus* individuals, these behaviours were recorded when they were directed towards other conspecifics. Sample sizes of observations for each of proximate and parallel swimming are as follows: *C. rainfordi* (n=28, both), *C. plebeius* (n=15, both), *C. baronessa* (n=36 and n=40, respectively), paired *C. lunulatus* (n=36, both), solitary *C. lunulatus* (n=16, both), *C. trifascialis* (n=15 and n=17, respectively), and *C. vagabundus* (n= 48 and 34, respectively). Proximate and parallel swimming with another conspecific in solitary *C. lunulatus* individuals was compared to that of paired *C. lunulatus* individuals using non-parametric Mann-Whitney *U*-test (SPSS Software), due to a lack of normality of residual variance. Sample sizes for observations of agonistic acts were as follows: *C. baronessa* (n= 50), *C. lunulatus* (n= 50), *C. vagabundus* (n= 50). (See **Table 2.1** for ethogram and sampling regime of all social acts recorded.)

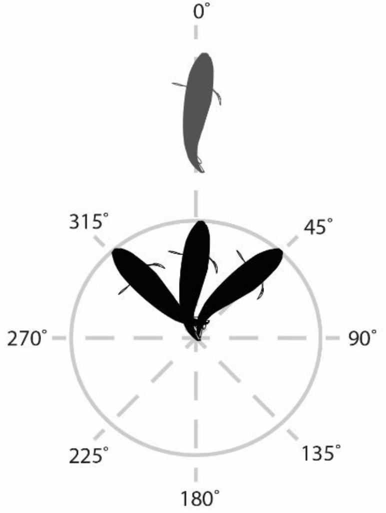


Figure 2.1. Schematic of parallel swimming examined in butterflyfishes. Parallel swimming by the focal fish (black) was defined as being faced within a 315-45° angle relative to the faced position of the conspecific (grey), whose faced position was designated 0°.

2.3.2.3 Homosexual versus heterosexual pairing

In order to determine whether pairs of butterflyfishes are predominantly heterosexual, a sub-sample of paired individuals (*C. baronessa,* n= 14 pairs; *C. lunulatus,* n= 14 pairs; *C. vagabundus*, n= 8 pairs) were collected following behavioural observations and sacrificed. Gonads were then removed and fixed in formaldehyde-acetic acid-calcium chloride (FACC) for at least 1 week. Thereafter, gonads were dehydrated in a graded alcohol series, cleared in xylene, embedded in paraplast, sectioned transversely (7 µM thick), and stained with

hematoxylin and eosin. Sections were examined under a compound microscope (400 X magnification) for the presence of sperm (male) or oocytes (female) (West, 1990; Cole and Hoese, 2001). To statistically test whether pairs are predominantly heterosexual, the number of heterosexual versus homosexual pairs (regardless of sex) was compared using a chi-squared homogeneity test in R (R Core Team, 2014).

2.3.2.4 Partner fidelity of pairs

To test for partner fidelity among pairs of butterflyfishes, I individually tagged up to 18 pairs of three different species (*C. baronessa,* n=12; *C. lunulatus,* n= 18; *vagabundus*, n= 17) and then re-surveyed tagged fishes repeatedly over 6-weeks to test whether initially paired individuals remained with their same partner throughout. To facilitate relocation of tagged fishes, this study was conducted on a single distinct platform reef, separated from nearby reefs by an open expanse of sand, which was expected to minimise movement of fishes away from the vicinity in which they were originally tagged. Paired fishes were identified as described above (section 1.3.2.3) and then caught using a barrier net. Paired individuals were tagged on opposite sides of the dorsal musculature with unique and matching colour coded external tags using a hand-held tagging applicator (Floy T-bar Anchor) (Berumen and Almany, 2009). After 1.5 months, a team of three snorkelers used an "expanding circle" search approach in order to relocate any tagged butterflyfishes. Once tagged fishes were located, 3-minute observations were conducted to test for partner affiliation, based on proximate and parallel swimming, and respective partners were then carefully examined to determine i) if they were tagged, and ii) the colour code of their external tags.

2.3.3 Examining the utility of the two-choice proximity assay for eliciting partner preference and selective agonism in the laboratory

2.3.3.1 Animal collection and housing

Pairs of *C. lunulatus* were collected using barrier nets on reefs on the western platform reefs and southern fringing regions of Lizard Island. Following collection, individuals of each pair were sexed via gonad catheterization (following Boyle and Tricas, 2014, and first described by Tricas, 1989) and then uniquely fin-clipped to differentiate individuals. The total length (TL) of all fishes was recorded, and fishes were given a two-minute fresh water bath to remove external parasites. Partners were kept together and placed within individual aquaria lined with sand, with dead coral for structural habitat and live fragments of *Acropora intermedia* for food. The day before trials, the focal male (n=11) and his partner were acclimated overnight in the central chamber of the testing tank (2.2 metre X 1.2 metre X 0.5 metre deep; volume: 1,000 liters). Trials were conducted

the following morning. All housing and experimental tanks had a continuous flow of fresh sea water, and ambient light and temperature.

2.3.3.2 Study assay: two choice proximity assay

A testing tank was divided into three chambers using clear mesh netting, allowing for transmission of visual, olfactory, and auditory cues; which are involved in conspecific recognition in butterflyfishes (Boyle and Tricas, 2014). The central chamber was further divided into three fully accessible zones that were created using string that was suspended above the water surface: two outer testing zones that were adjacent to either the partner's chamber or the non-partner's chamber, and one central zone (**Figure 2.4**). Each chamber was lined with sand, dead coral for structural habitat and live fragments of *Acropora intermedia* for food. Care was taken to ensure that these parameters, in addition to water flow, light level, and size of zone was equivalent among chambers. Just prior to experimentation, the focal male was placed in the central zone of the central chamber while his partner was placed into one of the side chambers, and a non-partner female of same sex and size (+/-2mm) as the partner was placed into the opposite side chamber, and allowed to acclimate for two hours. The side chamber in which each test female (left or right) was placed was chosen haphazardly. The trial then began by recording the position of focal males (relative to the three zones) every 30 seconds, and the number of agonistic acts directed towards its partner and non-partner throughout a one-hour observation period (see **Table 2.1** for ethogram of agonistic acts). Partner preference was determined by comparing total count data on male's position in partner versus non-partner zones using a paired *t*-test on ln transformed data. All behavioral observations were conducted from behind mesh shade cloth. Immediately after testing, focal pairs were returned to the reef from which they came.

Table 2.1. Ethogram and sampling regime of behavior used to characterize the social nature of paired and solitary groups among focal individuals (of C. lunulatus) and species of Chaetodon.

Social behavior	**Definition**	**Measured in relationship to**	**Sampling rate**
Proximate swimming (% time spent)	Swimming 0-1.5m distance from another conspecific	Pairs: Group member vs. non-group member Singletons: Non-group member	Once every 10 seconds
Parallel swimming (% time spent)	Face is positioned at 315-45˚ angle relative to conspecific's face position (designated 0°).	Pairs: Group member vs. nearest non-group member Singletons: Non-group member	Once every 10 seconds
Agonistic behavior[1]		Pairs: Group member conspecific vs. non-group member hetero- and conspecific	Total acts
Staring	Hovering and facing an opponent, with normal or tail-up postures		
Chasing	Swimming at full speed toward opponent(s), which swam away at full speed		
Fleeing	Swimming at full speed to leave opponent(s)		
Encircling	Swimming in front of the head of an opponent to circle it		
	Rises tail and lowers head, often with erected dorsal spines. The angle between the body axis and substratum varies from ~30-80˚		

Notes: [1] The agonistic behaviors studied here were first fully described in butterflyfishes by Yabuta (2000).

2.4 Results

2.4.1 Interspecific variation in sociality of sympatric butterflyfishes

2.4.1.1 Field observations of group size

In all six species, the distribution of individuals observed across different group sizes differed significantly from random (*C. baronessa*: χ^2=180, *P* <0.001; *C. lunulatus*: χ^2=265, *P*<0.001; *C. vagabundus*: χ^2 =108, *P* <0.001; *C. rainfordi*: χ^2 = 47, *P* <0.001; *C. plebeius*: χ^2 = 70, *P* <0.001; *C. trifascialis*: χ^2 =23, *P* <0.001). There was also a dichotomy in predominant group size across species. Regardless of study site, *C. baronessa, C. lunulatus*, and *C. vagabundus* had a predominant group size of two individuals, with 78, 84, and 71% of individuals found in pairs, respectively (**Figure 2.2**). Among these species, the other individuals of each of these species were mainly solitary; and a groups size of 3 were only ever observed for *C. lunulatus*, however this was only on one occasion. By contrast, *C . rainfordi, C. plebeius,* and *C. trifascialis* had a predominant group size of one individual, with 88, 90, and 80% of individuals found on their own, respectively (**Figure 2.2**). Individuals of these species were less commonly observed paired (10-15%), and very rarely observed in a group size of 3 (1-2%). Differences in species-typical group size do not co-vary with differences in phylogenetic relatedness (**Figure 2.2**). Group sizes exceeding 3 individuals were not observed.

2.4.1.2 Level of proximate and parallel swimming

The occurrence of proximate and parallel swimming clearly distinguished paired versus non-paired species (**Figure 2.3A, B**). Pairs of *C. baronessa, C. lunulatus* and *C. vagabundus* ranged as a single coordinated social unit throughout the reef, spending the majority of time (72 ± 7.41, 89 ± 6.2, and 81 ± 6.1 SE %, respectively) swimming within 1.5m from their partner, and most of the time (53 ± 8.1, 72 ± 5.8 SE, and 69 ± 6.6 SE %, respectively) were faced within a 315-45˚ angle of their partner. By contrast, singletons of *C. rainfordi, C. plebeius*, and *C. trifascialis* displayed no apparent social affiliation with another individual, as they spent 100 % of their time swimming further than 1.5m from another conspecific; and commonly, no other conspecific was within a field of view. Similarly, proximate and parallel swimming significantly and strongly varied between paired and solitary *C. lunulatus* individuals (proximate swimming: *U* = 9, *p* = 0.00; parallel swimming: *U* = 9.5, *p* = 0.00) (**Figure 2.3A, B)**. While paired individuals displayed these behaviors exclusively with their partners and at relatively high levels (swimming within 1.5m from partner for 89 ± 6.2 SE % of time; swimming faced within a 315-45˚ angle of their partner 72 ± 5.8 SE % of the time), solitary individuals displayed these behaviors at relatively low levels (swimming within 1.5m

from another conspecific 3.1 ± 2.3 % of time; swimming faced within a 315-45˚ angle of another conspecific 2.8 ± 1.5 % of the time).

2.4.1.3 Level of agonistic behavior among pairs

Pairs of *C. baronessa, C. lunulatus,* and *C. vagabundus* displayed agonism exclusively towards non-partners, as no level of agonism was observed towards pair members, and 100% of agonistic acts were directed towards non-pair members (**Figure 2.3C**). However, even agonism towards non-partners was infrequent and minor, consisting mostly of staring displays.

2.4.1.4 Partner endurance of paired groups

Across the three pairing species, a total of 29 of the original 47 tagged pairs were relocated after 1.5 months, and within the original general reef location. Seven out of 12 focal pairs of *C. baronessa,* 10/18 pairs of *C. lunulatus* and 9/17 focal pairs of *C. vagabundus* were re-identified at the end of the monitoring study (1.5 months post tagging). Among these re-identified pairs overall, partners appeared to have prolonged fidelity towards each other, as 5/7 (71%) of *C. baronessa,* 8/10 (80%) of *C. lunulatus*, and 8/9 (89%) of *C. vagabundus* pairs were grouped with their original partner (**Figure 2.3D**). In three of the cases where focal fishes were not re-located with their original partner, the focal fishes were found alone; whereas in the other two cases, the focal fish was found paired with another non-tagged fish. The missing original partners of these five focal individuals were not found.

2.4.1.5 Sex composition of paired groups

Most (but not all) of the pairs of *C. baronessa, C. lunulatus,* and C. *vagabundus*, for which we determined sex histologically, were heterosexual. The ratio of heterosexual pairs differed significantly from a uniform distribution (0.5; binomial test, $p < 0.05$ for each species), with a ratio of 0.86 heterosexual pairs of *C. baronessa* (n = 12/14), 0.93 heterosexual pairs of *C. lunulatus* (n = 13/14), and of 1.0 heterosexual pairs of *C. vagabundus* (n = 8/8). The two homosexual pairs of *C. baronessa* were female-female, while the one homosexual pair of *C. lunulatus* was male-male.

2.4.2 Functional design: Validating utility of the partner preference assay

In the two-choice proximity assay used to assess partner preference, male *C. lunulatus* were strongly motivated to affiliate with one or other females, spending most their time positioned within the zones proximal to them (54/60min ± 1.5 SE), and minimal time in the central zone (6/60min ± 1.5 SE). Males displayed similar affiliative behavior towards both females, often positioning themselves directly adjacent to females and displaying coordinated swimming movement with them, which was reciprocated by females. Males generally spent large amounts of time within a given preference zone, and showed minimal transitory movement between preference zones. However overall, the assay appeared to elicit strong partner preference in males, as in eight out of 11 (72%) cases, males spent considerably and significantly more time in the zone adjacent to their partner (48/60min, ± 3.5 SE) than in the zone adjacent to the non-partner conspecific (6/60min ± 1.8 SE) ($t = 5.41$; $df = 7$, $p = 0.001$) (**Figure 2.4B**). The three males that did not display partner preference, displayed non-partner preference, spending on average (37/60min ± 8.5 SE) in the zone adjacent the non-partner, and only (16/60min ± 7.1 SE) in the zone adjacent to their partner. This was associated with the non-partner female displaying notably more swimming activity than the partner female. No agonistic behavior was observed in any test fish in the study.

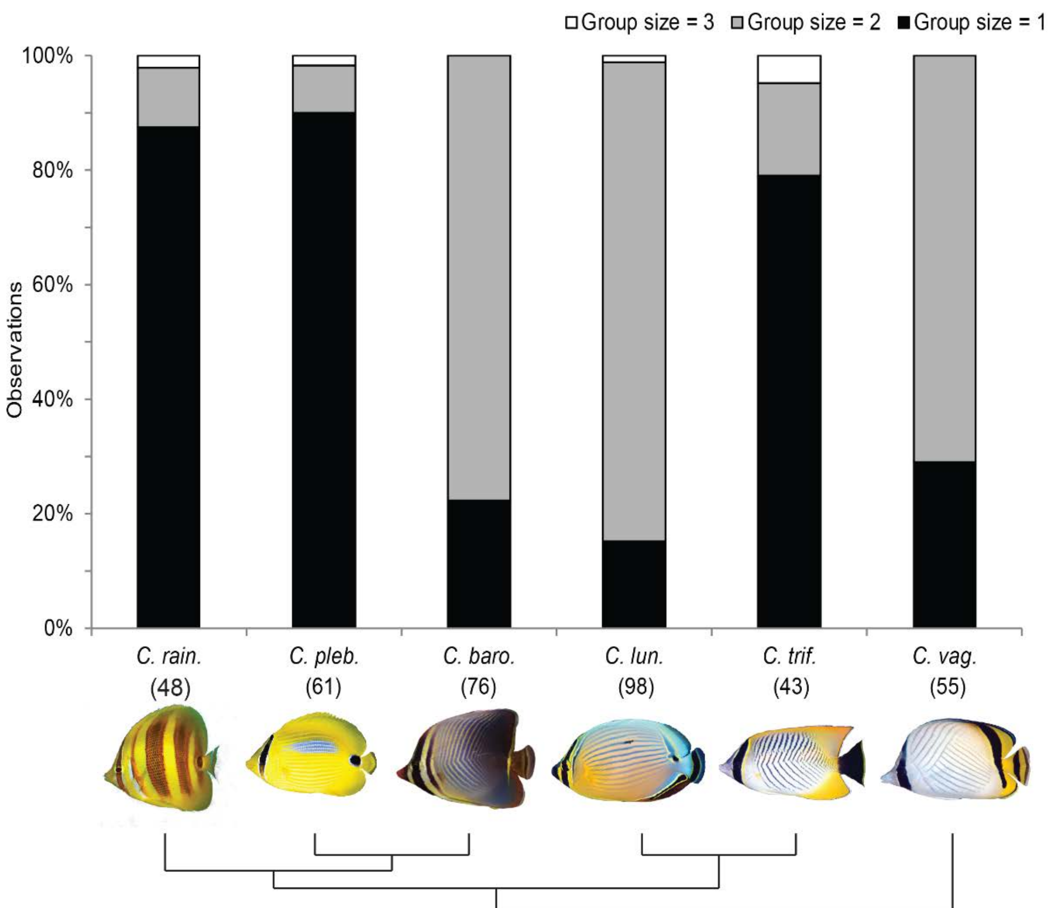


Figure 2.2. Group size frequency distribution and phylogenetic relationships of six congeneric butterflyfish species at Lizard Island. Numbers in parentheses indicate sample sizes. Phylogeny re-drawn from Bellwood et al., 2010; Cowman and Bellwood, 2011.

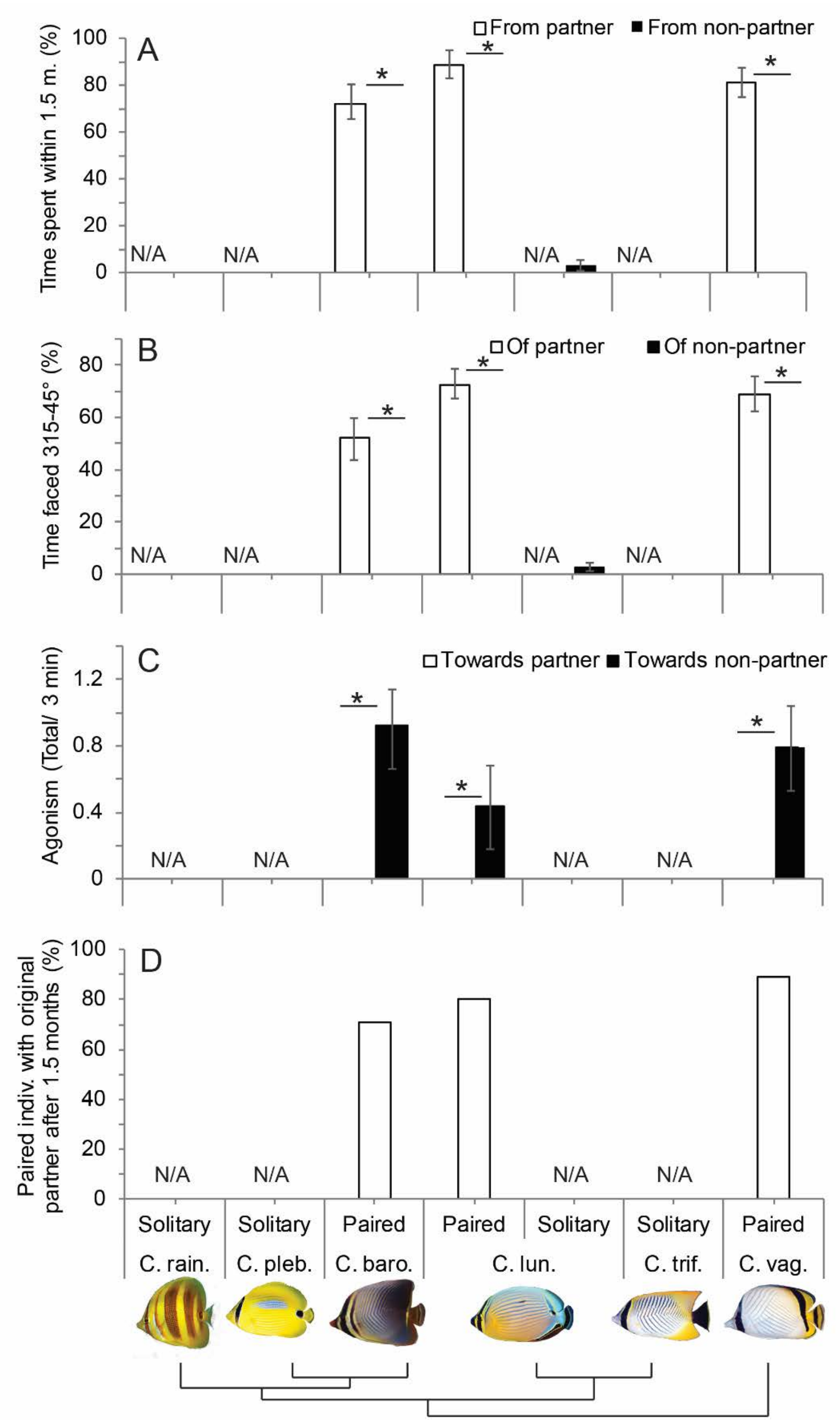
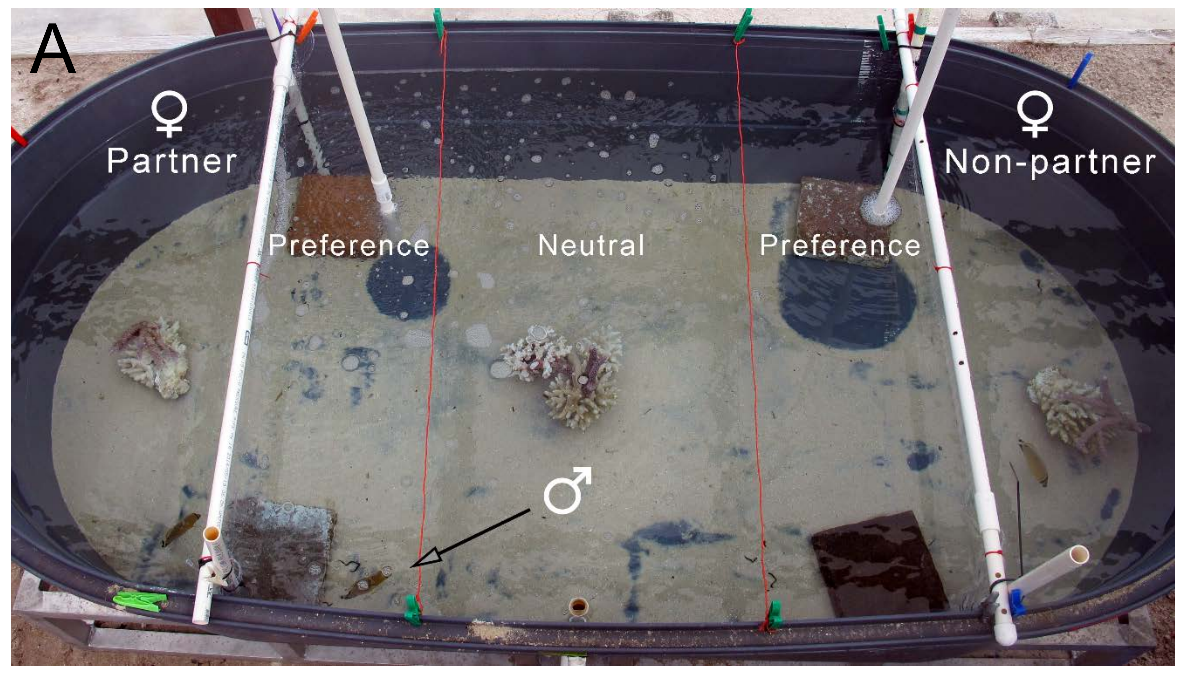


Figure 2.3. Differences in social behaviors and phylogenetic relationships between predominantly paired and solitary grouped butterflyfish species and between paired and solitary *C. lunulatus* individuals. (A) = Mean ±SE % time spent proximate swimming with another conspecific. (B) = Mean ±SE % time spent parallel swimming with another conspecific. (C) = Mean ±SE antagonism towards partner vs. non-partner among pairs. (D) = Percentage of pairs displaying partner fidelity after 1.5 months. Asterisks indicate significant differences between groups. Phylogeny re-drawn from Bellwood et al., 2010; Cowman and Bellwood, 2011.



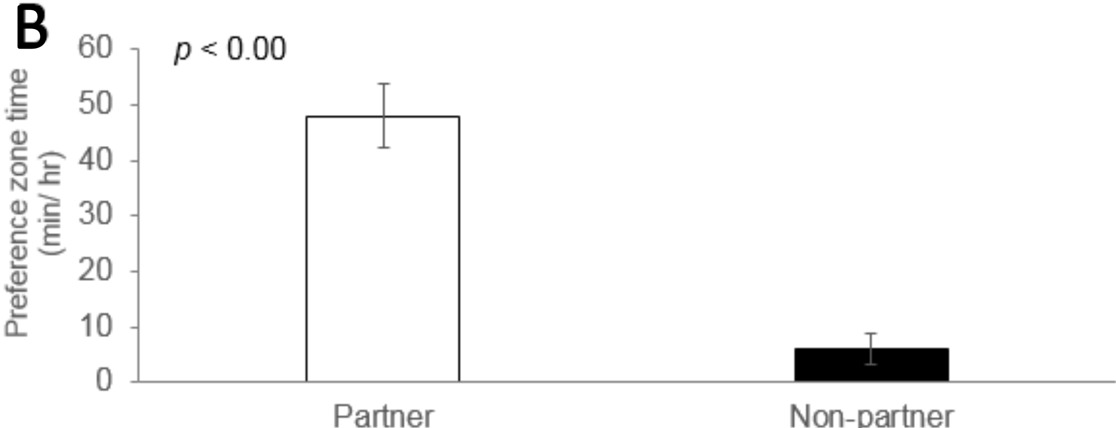


Figure 2.4. The two-choice proximity assay elicited partner preference in *C. lunulatus* males. Males were placed in a central "choice chamber", with their partner female and a non-partner female on either end. Following, time spent in the "preference" zone adjacent to either test female (delineated by red string above tank) was quantified (**A**). Time spent (mean ± SE) in the partner zone was significantly higher than in the non-partner zone (**B**).

Table 2.2 Dichotomous sociality (pair bonding vs. solitary living) on an individual-level (in *Chaetodon lunulatus*) and species-level at Lizard Island (found in current study) does not co-vary with other attributes, making these populations useful for highly controlled comparative studies on pair bonding.

Species	Social system	Clade	Geographic occurrence	Parental care	Feeding ecology	Territoriality	Mating system	References
C. rainfordi	**Solitary**	C3	Sympatric	None	Coral	Low	Unknown	1,2,3,5
C. plebeius	**Solitary**	C3	Sympatric	None	Coral	Low	Unknown	1,2,3,5,6
C. baronessa	**Pair bond**	C3	Sympatric	None	Coral	High	Unknown	1,2,3,5,6
C. lunulatus	**Pair bond**	C3	Sympatric	None	Coral	Moderate	Monogamy**	1,2,3,4,5,6,7
	Solitary		Sympatric	None	Coral	Moderate	Unknown	

C. trifascialis	**Solitary**	C3	Sympatric	None	Coral	High	Polygyny**	1,2,3,5,6,8
C. vagabundus	**Pair bond**	C4	Sympatric	None	Coral and Non-coral	Low	Unknown	1,2,3,5

References: 1: current study; 2: Roberts and Ormond, 1992; 3: Pratchett, 2005; 4: Gregson et al., 2008; 5: Berumen and Pratchett, 2006; 6: Blowes et al., 2013; 7: Yabuta, 1997; 8: Yabuta and Kawashima, 1997. Phylogeny data from Bellwood et al., 2010, and Cowman and Bellwood, 2011.

Notes: * Although parental care has not been studied in Lizard Island butterflyfish populations, it is presumed to be absent based on unequivocal reporting of pelagic spawning within the family.

**The mating systems of these populations are presumed based on reports at other populations (Yabuta, 1997; Yabuta and Kawashima, 1997).

2.5 Discussion

2.5.1 Comparative analyses: Inter- and intra-specific variation in sociality for Chaetodon *butterflyfishes*

This study formally characterized the sociality of six common and widespread species of coral reef butterflyfishes, largely reaffirming the previously assumed social (and mating) systems that were mostly based on observations of predominant group size (e.g., Reese, 1975; Yabuta, 2006; Pratchett et al., 2006). Three of the focal species, *Chaetodon baronessa, C. lunulatus,* and *C. vagabundus*, are generally assumed to be not only pair bonding, but also monogamous (Whiteman and Côté, 2004). Field servys revealed that individuals of these species were predominantly found in social groups of two individuals, consistent with previous studies (Reese, 1975; Bouchon-Navaro, 1981; Yabuta, 2007). By contrast, 88, 90, and 80 % of individuals of *C. plebeius, C. rainfordi,* and *C. trifascialis*, respectively, were recorded as singletons, only rarely occurred paired, consistent with previous reportings (Reese, 1975; Bouchon-Navaro, 1981; Fricke, 1986; Yabuta, 2007).

2.5.1.1 Intra-specific variation in sociality for Chaetodon lunulatus

Chaetodon lunulatus is routinely assumed to be pair bonding, mostly because it occurs predominantly in pairs (Yabuta and Berumen 2014). However, for this species, at least, there is substantial additional evidence that these species do form enduring pro-social affiliations, which also extends to monogamous mating (Yabuta, 1997). In the Yaeyama Islands, for example, pairs of *C. lunulatus* spend 84% of their time within close proximity to each other, with spawning occuring exclusively between established pairs (Yabuta, 1997). The reproductive basis of pairing in *C. lunulatus* is further supported by concurrence between the size at which fishes formed pairs and the size specific onset of sexual maturity (Pratchett et al. 2006). Pairs of *C. lunulatus* have also been reported to persist for at least seven years (Reese, 1991; Yabuta, 1997; 2000). Similarly, in this study I found that the majority of *C. lunulatus* form pairs (90 % of individuals), most of which are heterosexual (93% of pairs), and that are further characterized by a high level of exclusive proximate and parallel swimming with partners (89% and 72% of time, respectively) and exclusive agonism towards non-partners. All but 2 tagged pairs of *C. lunulatus* persisted throughout the duration of the study (1.5 months). While I had hoped to measure partner fidelity for much longer time period (> 12 months), this was not feasible, due to excessive algae growth on and loss of tags after 1.5 months. In future studies, I suggest using unique markings on focal individuals for identification (Yabuta, 1997; Chapter 4) in preference to external tags.

Despite the predominance of pairing among mature individuals of *C. lunulatus* observed at Lizard Island, and elsewhere (Reese, 1973, 1975, 1991; Sutton, 1985;

Yabuta, 1997, 2000, 2002), there are a significant number of mature individuals that do not occur in pairs. At Lizard Island, I recorded 15% of adult *C. lunulatus* as solitary individuals. Elsewhere, the proportion of solitary individuals for *C. lunulatus* may be as high as 47% (Reese 1975). In any population, a small proportion of mature individuals would be expected to be solitary due to either a scarcity of suitable partners or the loss of partners (e.g. Harding et al. 2003). However, it is also possible that there are individual differences in the propensity to form pairs, which is very useful for exploring mechanistic correlates of pair bonding for butterflyfishes (See Chapter 3).

2.5.1.2 Inter-specific variation in sociality

Like *C. lunulatus*, *C. baronessa* and *C. vagabundus* also exhibit high prevalence of pairing, though pair bonds are generally weaker, such that the prevalence of pairing is less conspicuous (Reese, 1975). While never explicitly tested, the prevalence of pairing does suggest monogamous mating (Yabuta and Berumen 2014). Accordingly, I found that *C. baronessa* and *C. vagabundus* were predominantly found in heterosexual pairs that displayed agonism exclusively towards non-partner hetero- and con-specifics. Although agonism was exclusively directed towards non-partners, it was generally passive (i.e., dominated by visual displays and chase rates were low). This has been previously reported in territorial butterflyfish (Reese, 1975; Tricas, 1985), and is consistent with the 'dear enemy' model of low-cost resource defense once territories have been established among neighboring pairs (Wilson, 1975; Tricas, 1989). Pairs displayed strong partner fidelity, with all but two pairs of *C. baronessa* and one pair of *C. vagabundus* lasting throughout the duration of the study (1.5 months). Partner fidelity has only been previously studied in *C. baronessa,* where focal pairs remained together for at least 4 months (Reese, 1973). I conclude that (as for *C. lunulatus*)*, C. baronessa* and *C. vagabundus* are strongly pair bonding at Lizard Island. However, observations of spawning for specific focal fishes (as per Yabuta 1997) will be required to establish whether mating occurs exclusively between paired individuals (**Table 2.2**).

An altogether different social system is found in *C. plebeius, C. rainfordi,* and *C. trifascialis*, whereby individuals rarely occur within close proximity (and certainly never exhibit proximal swimming) with conspecifics. For *C. trifascialis*, Yabuta and Kawashima (1997) established that large males typically have large territories encompassing the entire territories of one or more females. The males repeatedly visit each of the females within their territories, but only spend a short time swimming together (Yabuta and Berumen 2014), which would explain the occasional sighting of seemingly paired *C. trifascialis* at Lizard Island (**Figure 2.2**). Yabuta and Kawashima (1997) further demonstrated that male *C. trafascialis* mate sequentially with each of the females contained within their territories, suggestive of an haeremic mating system. The mating systems of the other species are however, yet to be established (**Table 2.2**). Despite

limited occurrence of pairs, *C. plebeius* and *C. rainfordi* (but not *C. trifascialis*) have been considered by some researchers to be monogamous (Whiteman and Côté 2004: Table 2). More likely is that *C. rainfordi* (like *C. trifascialis*), is polygynous, whereas more work is required to establish the mating system for *C. plebeius* (Yabuta and Berumen 2014).

The sociality of butterflyfishes is yet to be explicitly mapped on to well-established phylogenies available for these fishes (e.g., Fessler and Westneat, 2007; Bellwood et al., 2010; Cowman and Bellwood, 2011), mostly because more work is still required to resolve the sociality of many key groups. Hence, it is not possible to establish, whether pair bonding evolved independently among *Chaetodon* butterflyfishes or results from a single common (and presumably pair bonding) ancestor. Preliminary assessment, based on the sociality of the six study species (*C. baronessa, C. lunulatus, C. plebeius, C. rainfordi C. trifascialis*, and *C. vagabundus*) considered in this study, tentatively suggests that pair bonding might be an ancestral trait, while the other socialites are more derived. Molecular phylogenies show that 5/6 study species are closely related congeners of the genus *Chaetodon*, belonging to a single clade (C3) while *C. vagabundus* belongs to the sister clade C4 (Fessler and Westneat, 2007; Bellwood et al., 2010; Cowman and Bellwood, 2011). Using outgroup criterion on this limited available data (**Figure 2.2**), the most parsimonious explanation for appearance of pair bonding across the six study species, is that the common ancestor of all six study species exhibited pair bonding. Importantly, this would tentatively imply that the three pair bonding species are ill-suited for studying whether convergence in pair bonding within the group has been produced by convergent governing mechanisms (Goodson and Kingsbury, 2011). Rather, they'd be better suited for generating insight into whether the conservation of pair bonding among sister species is a product of conserved mechanistic underpinnings. What is clear, is that ancestral character reconstruction is an important next step in developing the butterflyfish model system, and specifically in resolving the evolutionary history of sociality among its constituent species.

2.5.1.3 Further considerations for comparative designs: additional confounding factors

Although many species of fishes are pair bonding, and several families display spectacular interspecific variation in sociality (Whiteman and Côté, 2004; Brandl and Bellwood, 2014), very little is known about the regulatory mechanisms that underpin this phenomenon. Recently, comparative designs have been developed in cichlids and butterflyfishes, and have generated informative preliminary mechanistic insights (Dewan et al., 2011; Oldfield et al., 2013; O'Connor et al., 2016). One drawback of the current cichlid systems, however, is that pair bonding comparisons are restricted to just two species and are confounded with several other attributes (see *further considerations*, below). The pre-established butterflyfish design compares several

(seven) species, and more independently of other attributes (i.e., species relatedness, territoriality, and mating system); however, this comparison is in respect to social group size (Dewan et al., 2011). While social group size is a fundamental component of butterflyfish social systems, it does not fully characterise and therefore validate them (Reese, 1975; Hourigan, 1989; Roberts and Ormond, 1992; Yabuta and Berumen, 2014). Hence, a more controlled, validated, and reliable system for comparatively studying species variation in pair bonding is warranted.

Neural mechanisms that govern pair bonding (and social systems, in general) independently of other behavioral or ecological domains are poorly understood (but see Goodson et al., 2005, 2006). This might be due to the fact that in most vertebrate groups, sociality often co-varies with several other behavioral attributes (Goodson et al., 2006). For example, even in the most widely-used model systems for comparatively studying pair bonding, the *Microtus* and *Permyscus* voles, species differences in social system are confounded with parental care, habitat preference, and mating systems (Goodson et al., 2006; Goodson and Kingsbury, 2011). There are two existing cichlid model systems for examining species differences in pair bonding. In the *Herichthys* cichlid system, males of species that differ in their pair bonding behaviour also differ in their territoriality, the extent to which they contribute to parental care, and their mating system (Oldfield et al., 2013). In the *Neolamprologus-Telmatochromis* system, species differences in pair bonding strength are confounded with differences in group living and overall sociability (O'Conner et al., 2015, 2016). Confounding pair bonding with social bonding, parental care, and territoriality is especially problematic, because shared mechanisms (particularly neuroendocrine mechanisms) have been shown to independently regulate all of these attributes (Goodson and Kingsbury, 2011; Goodson, 2013; Oldfield et al., 2015).

Butterflyfishes provide an excellent opportunity to eliminate effects of phylogeny, behavior, ecology that commonly confound interspecific comparisons of pair bonding (Dewan et al., 2011), owing to their spectacular diversity in these domains. While the six species chosen in the current study (*C. baronessa, C. lunulatus, C. plebeius, C. rainfordi C. trifascialis*, and *C. vagabundus*) clearly display variation in their social systems, several aspects of their behavioral ecology, biogeography, and phylogeny do not co-vary (**Table 2.2**). These species, as in all butterflyfishes, are exclusively pelagic spawners, so do not display any parental care (Lobel, 1978; Neudecker and Lobel, 1982; Thresher, 1984). All species are sympatric at Lizard Island, and are benthic feeders that (with the exception of *C. vagabundus*) feed mainly on scleractianin corals (Pratchett, 2005; Lawton et al., 2012). Although relatedness and territoriality varies among species (Berumen and Pratchett, 2006; Blowes et al., 2014), this is a manner that is inconsistent with variation in social system. However, mating systems, while yet to be verified, are presumed to co-vary with species differences in social systems, such that pair bonded

species are expected to be reproductively monogamous, and non-pair bonded species are expected to be reproductively promiscuous/polygamous. Notably, all of these aforementioned controls also apply for pair bonded vs. solitary individuals of *C. lunulatus* (**Table 2.2**). Hence, the proposed designs offer a rare opportunity for highly controlled intra- and inter- species comparative research on teleost pair bonding.

2.5.2 Functional analyses: Validating the utility of the two-choice proximity assay

My field observations showed that exclusive proximal affiliation with a partner is a ubiquitous feature of pair bonding butterflyfish, making it an ecologically relevant proxy for pair bonding in these organisms. Moreover, butterflyfishes exhibit overt behavioral displays (both negative and positive) towards conspecifics (Hourigan, 1989; Strang, 2005; Yabuta, 1999, 2000, 2002) that can be easily observed in wild populations and are maintained in laboratory setting (Dewan personal observation). This rich and, more importantly, well characterized social behavior, provides a strong foundation for studies focused on proximate mechanisms of behavioral modulation. For example, the frequency of individual visual displays of agonism was recently correlated to features of the AVT system in a territorial butterflyfish (Dewan and Tricas, 2011).

In several taxa, partner preference, broadly characterized by selective affiliation with a partner over a non-partner (Young et al., 2011), is a quantifiable feature of pair bonding that can be experimentally elicited using two-choice proximity assays (e.g., rodents: Williams et al., 1992; Winslow et al., 1993; birds: Swaddle and Page, 2007). Hence, this behavioral proxy and assay are effective for experimentally studying mechanisms of pair bonding in these organisms (Williams et al., 1992). However, in marmosets, paired individuals spend more time near an unfamiliar conspecific when in a paradigm similar to the two-choice proximity assay (Smith et al., 2010). Likewise, in male king quails, proximity to partner within the two-choice proximity assay is an invalid way to assess selectivity of pairing behavior because it is often accompanied by aggressive behavior (Adkins-Regan, 2016). Hence, in some species, using proximity alone to assess selective affiliation in the two-choice proximity assay is useful; whereas in others, other affiliative and agonistic behaviors, in addition to proximity, are needed for valid assessment of selective affiliation.

This study shows that *C. lunulatus*, which displayed the strongest levels of pair bonding in the field, exhibits preferential affiliation for partner in two-choice proximity assays. In all, eight (out of 11) males tested displayed significant partner preference, spending a disproportionate amount of their time positioned within close proximity of their partner. This was accompanied by coordinated swimming activity and an absence of agonism, indicating that the proximal behavior represented an affiliative association. Three males, however, spent a disproportionate amount of time within close proximity

of non-partner females, where these females exhibited much higher activity levels than partner females. These findings indicate that the relative activity levels of partner versus non-partner females may confound estimates of affiliation based solely on proximity. Nonetheless, my results do show that on average, *C. lunulatus* males who are in pre-established relationships display significant partner preference, demonstrating the utility of this study species and the modified two-choice proximity assay for experimentally assessing the functional basis of pair bonding in marine fishes. In contrast to my findings, paired males the congener, *C. multicinctus*, spend more time with unfamiliar individuals than partners when given a choice in the wild, based on visual and olfactory cues of both stimulus fish (Boyle and Tricas, 2014). However, given that this occurs when unfamiliar individuals are placed within the male's residence, and coincides with agonistic displays, such preferential proximity is likely representative of territorial behavior rather than affiliation.

In addition to selective affiliation for a partner, selective agonism towards non-partner conspecifics of an opposite sex is also hallmark of pair bonding in male prairie voles (Winslow et al., 1993) and king quails (Adkins-Regan, 2016). My field study revealed that selective agonism towards non-partner conspecifics is also an ubiquitous characteristic among the three species of pair bonding butterfyfishes. However, unlike in the king quail (Adkins-Regan, 2016), selective agonism towards a non-partner conspecific female was not elicited by the laboratory two-choice proximity test in *C. lunulatus* males. There are several potential explanations for these findings. First, in butterflyfishes, conspecific agonism is strongly attributed to feeding territory defense (Reese, 1975; Sutton, 1985; Fricke, 1986; Berumen and Pratchett, 2006; Blowes et al., 2014). Since males were tested outside of their pre-established territories in the wild and given limited time to potentially establish a new territory in captivity (overnight), selective agonism that might have been attributed to territorial defense may have been absent in this study. Secondly, limited field observations further suggest that in pair bonding butterflyfishes, conspecific agonism is only directed towards same-sexed individuals (Fricke, 1986). Therefore, an experimental paradigm that includes the presence of an established territory (e.g., a resident intruder paradigm (Winslow et al., 1993; Boyle and Tricas, 2014)), and/or examines agonism towards same-sexed conspecifics might be more ecologically relevant and hence reliably elicit selective agonism in this organism.

2.5.3 Conclusions

This study has demonstrated the utility of *Chaetodon* butterflyfishes for both comparative and functional analyses of the neural basis for pair bonding (and other social systems). This is important both to establish the neurobiology of sociality in fishes, as well as understanding the origin and evolution of specific neurological systems that

facilitate pair bonding in vertebrates. There are nonetheless, several challenges and limitations to ongoing research in these organisms. First, unlike most established model systems (e.g., voles and freshwater fishes), *Chaetodon* butterflyfishes are difficult to breed and maintain in captivity (Delbeek, 2014), largely due to their highly specialized dietary and habitat requirements. We found that even when maintaining *C. lunulatus* in captivity for very short periods (24 hours), it is virtually impossible to provide sufficient abundance and diversity of live corals to meeting their nutritional requirements. Using pair bonding non-corallivorous butterflyfishes may be a tractable solution to this problem, but the feeding generalist *C. vagabundus* is much less abundant, and exhibits weaker pair bonding compared to *C. lunulatus*. Therefore, functional analyses will need to be conducted with wild caught butterflyfishes, while limiting the period that these fishes are maintained in captivity (Chapter 3).

Coral reef fishes exhibit highly diverse forms of sociality (Sale, 1978). When overlaid on a detailed phylogeny, numerous hypotheses relevant to the origin and evolution of sociality can be explicitly tested. Coral reef fish families in which closely related species exhibit diverse social systems, as shown for *Chaetodon* butterflyfishes, are a powerful tool to test these hypotheses. However, much more research is needed to explicitly resolve the specific social and mating systems across a much broader range of *Chaetodon* species, in order to enable more robust and controlled comparative analyses.

Chapter 3: Neurobiology of pair bonding in fishes; convergence of neural mechanisms across distant vertebrate lineages

3.1 Abstract

While pair bonding has independently evolved numerous times among vertebrates, the governing neural mechanisms of pair bonding have only been studied in depth in the mammalian model species, the prairie vole, *Microtus ochrogaster*. In this species, oxytocin (OT), arginine vasopressin (AVP), dopamine (DA), and opioid (OP) systems play key roles in signaling in the formation and maintenance of pair bonding by targeting specific brain regions. By contrast, the neural basis of pair bonding is poorly studied in other vertebrates, and especially those of early origins, limiting our understanding of the evolutionary history of pair bonding regulatory mechanisms. I used a classic partner preference assay to determine whether these well-established mammalian neurochemical systems (or their ancestral homologs) govern pair bonding in the monogamous, non-parental coral reef butterflyfish, *Chaetodon lunulatus*. Male *C. lunulatus* were administered either receptor antagonists or saline, to test for changes in selective affiliation with partners. OT-like (isotocin) receptor (ITR), and AVP-like (arginine vasotocin) receptor (AVT V1aR) antagonists significantly reduced the percentage of time males spent affiliating with their partner; whereas the dopamine D1 receptor (D1R) and mu-opioid receptor (MOR) antagonists produced no significant effect. I then compared receptor gene expression between pair bonded and solitary individuals across eight key brain regions. I found that in females, ITR and V1aR receptor expression varied in the lateral septum-like region (the Vv/Vl), while in both sexes D1R, D2R, and MOR expression varied within the mesolimbic reward system, including a striatum-like region (the Vc); mirroring sites of action in *M. ochrogaster*. This study provides novel insights into the neurobiology of teleost pair bonding. It also reveals high convergence in the neurochemical mechanisms governing pair bonding across actinopterygians and sarcopterygians, by repeatedly co-opting and similarly assembling deep neurochemical and neuroanatomical homologies that orginated in ancestral vertebrates.

3.2 Introduction

Pair bonding has independently evolved in all major vertebrate lineages (Reichard and Boesch, 2003), where it represents a major defining feature of species-specific social structure (Goodson and Kingsbury, 2011) including that of humans' (Quinlan and Quinlan, 2007; Quinlan, 2008; Fletcher et al., 2015). As such, the neural basis of pair bonding in mammals is particularly well-studied (reviewed by: Carter et al., 1995; Aragona and Wang, 2004; Young and Wang, 2004; McGraw and Young, 2010; Young et al., 2011; Freeman and Young, 2013; Johnson and Young, 2015; Gobrogge and Wang,

2016), largely due to its translational implications for the mechanistic underpinnings of human pro-sociality (e.g., "romantic love")(Carter, 1998; Young and Wang, 2004; Zeki, 2007; Young, 2009; Freeman and Young, 2016), and conversely, for better understanding and treating anti-social psychiatric disorders (Volkmar, 2001; Aragona and Wang, 2004; Freeman and Young, 2016).

Most of what is known about the neural basis of mammalian pair bonding comes from extensive research on a small rodent, the prairie vole, *Microtus ochrogaster*. In this species, oxytocin (OT), arginine vasopressin (AVP), dopamine (DA), and opioid (OP) neurochemical systems play key interactive roles in signaling the formation and maintenance of pair bonding (reviewed in Young and Wang, 2004; Young et al., 2011; Johnson and Young, 2015; Donaldson and Young, 2016; Numan and Young, 2016). In females, sociosexual activity triggers OT release, which acts on OT receptors (OTRs) in the striatal nucleus accumbens (NAcc) and the prefrontal cortex (PFC) (Young et al., 2001; Liu and Wang, 2003); thereby promoting partner preference. AVP systems also regulate female partner preference formation (Cho et al., 1999); however, targeted brain regions remain unknown. In males, both OT and AVP nonapeptide systems also appear to mediate mating-induced partner preference formation (Cho et al., 1999; and see Numan and Young, 2016 reference to Keebaugh et al., unpublished data). This likely involves OT-OTR signaling within the medial PFC (see Numan and Young, 2016 reference to Keebaugh et al., unpublished data), while it requires both OT-OTR and AVP-V1aR signaling in the NAcc- ventral pallidum (VP) circuitry (Numan and Young, 2016), and AVP-V1aR activity in the lateral septum (LS) (Liu et al., 2001). AVP-V1aR also promotes mating-induced selective non-partner aggression (mate-guarding) in males (Winslow et al., 1993; Young et al., 1997; Cho et al., 1999) at least partially by signaling within the anterior hypothalamus (Gobrogge et al., 2007, 2009). These aforementioned nonapeptide behavioral effects may result, at least partially, from their more general roles in regulating individual recognition; which may occur through concurrent OTR and olfactory signaling within the medial amygdala (MeAMY) (Lim and Young, 2004; Numan and Young, 2016); however, this is yet to be empirically tested. Different dopamine receptor sub-types appear to be involved in pair bond formation and maintenance. In both sexes, NAcc D2R activation promotes pair bond formation (Gingrich et al., 2000; Aragona et al., 2003, 2006); and in turn, pair bond formation subsequently up-regulates NAcc D1R activity, promoting selective aggression towards, and thus inhibiting pair bond formation with, other prospective partners (Aragona et al., 2006; Resendez et al., 2016). This D1R regulation of selective aggression is mediated by downstream activation of kappa-opioid receptors (Resendez et al., 2016) (see below). The source of DA projections to the NAcc in these pathways is the ventral tegmental area (VTA) (Gobrogge and Wang, 2009). As with dopamine, different opioid receptor sub-types appear to selectively mediate either pair bond formation or maintenance. Specifically, mu-opioid receptors (MORs) within sub-structures of the striatum (ie., the caudate putamen (CP), dorsal striatum, and dorsomedial NAcc shell) regulates pair bond formation (Burkett et

al., 2011; Resendez et al., 2013). Dorsal striatum MORs achieve this through regulating mating, while dorsomedial NAcc shell MORs appear to achieve this through mediating positive hedonics associated with mating (Resendez et al., 2013). Finally, kappa-opioid receptors (KORs) within the NAcc shell regulate pair bond maintenance (Resendez and Aragona, 2013; Resendez et al., 2012, 2016) by mediating aversive social motivation (Resendez et al., 2012, 2016). Because very little is known about the neurobiology of pair bonding in other species, and especially those of earlier evolutionary origins: reptiles (Bull, 2000), amphibians (Brown et al., 2010; Roland and O'Connell, 2015), and fishes (Whiteman and Côté, 2004; Brandl and Bellwood, 2014) (but see Dewan and Tricas; 2011, Dewan et al., 2011, Oldfield and Hofmann, 2011, O'Connell et al., 2012), the evolutionary history of neural circuitry governing vertebrate pair bonding remains poorly understood.

Convergence of evolutionarily labile traits, especially across remotely related lineages has been traditionally thought to be underpinned by entirely different regulatory processes (Butler and Hodos, 2005). In the case of vertebrate pair bonding, there have been literally hundreds of independent transitions (e.g., there have been at least 61 among mammals (Lukas and Clutton-Brock, 2013), and 13 among coral reef fishes (Brandl and Bellwood, 2014)), that have occurred across taxon that are separated by up to 450 million years of independent evolution (actinopterygians and sarcopterygians) (Kumar and Hedges, 1998). Hence, it is conceivable that the far-ranging convergence of vertebrate pair bonding may be a product of entirely different regulatory systems being selected upon in order to achieve the same phenotypic outcome (Goodson and Thompson, 2010; Goodson, 2013). This may be especially the case for nonapeptide involvement, since these systems are expected to evolve in very species-specific ways, depending upon the evolutionary background of the species (Goodson, 2013). Indeed, while the role of nonapeptides in governing pair bonding in *M. ochrogaster* is well established, evidence for their involvement in certain other species has been absent. Among eight species of *Peromyscus* mice, there is no association between pairing sociality and V1aR density within the VP, nor within other brain regions examined (Turner et al., 2010). In male zebra finches, *Taeniopygia guttata*, AVT V1a-like binding sites within the VP are of low density (Goodson et al., 2006), and central administration of AVP V1R antagonist cocktail does not affect pair bond formation (Kabelik et al., 2009). With regards to the OT-like system, in *Peromyscus* mice, NAcc OTR expression is not associated with species differences in pairing sociality (Insel et al., 1991); and pairing finches, *T. guttata,* and sparrows, *Zonotrichia albicollis,* exhibit no detectable expression of OTRs (or binding sites) in the NAcc nor the surrounding striatum (Leung et al., 2009, 2011).

Key neuro-chemical components of *M. ochrogaster* pair bonding have ancient evolutionary origins, as they were already established in the last common ancestor of ray- and lobe-finned fishes (ancestral osteichthyes) ~450 MYA. Their structure and

functions have since remained highly conserved across vertebrates (**Figure 3.1**). Vertebrate nonapeptides all derived from arginine vasotocin (AVT), which originated in jawless fishes (agnathans) ~500 MYA (Archer et al., 1995). In early jawed fishes (gnathostomes), the AVT gene duplicated (Archer et al., 1995), giving rise to two lineages, AVP- and OT-like nonapeptides. In the AVP-like lineage, AVT has remained present in all non-mammalian species; whereas, a single amino acid substitution was made in AVT in most mammals, giving rise to AVP. In the OT-like lineage, the gene duplication event in early jawed fishes gave rise to isotocin (IT), which is found in all extant bony fishes (teleosts). Prior to water-land transition, IT was replaced by mesotocin (MT), which is mostly present in extant lungfish, amphibians, reptiles, and birds (Goodson, 2008). Finally, MT was replaced by OT in most mammals (Archer, 1972; Hoyle, 1998; Lee et al., 2011). Cartilaginous fishes have evolved at least six OT-like peptides, including the mammalian OT form (Archer et al., 1999). Despite these alterations with the nonapeptide family, OT and AVT differ by only one amino acid (Archer and Chauvet, 1988). Nonapeptides play fundamental roles in regulating social behavior and physiology in all vertebrate taxa (Goodson and Thompson, 2010). Dopamine and the two major classes of dopamine receptors (D1 and D2Rs) pre-date the origin of chordates, and have since remained highly conserved across the phylum (Callier et al., 2003; Yamamoto and Verneir, 2011). The dopamine system serves a diverse array of behavioral and physiological functions, some of which, including associative reward learning, are shared across different lineages (Barron et al., 2010; Yamamoto and Verneir, 2011). The opioid system, primarily consisting of three endogenous ligands (endorphins, enkephalins, and dynorphins) and their conjugate receptors (mu-, кappa-, and delta-receptors) (Dreborg et al., 2008; Le Merrer et al., 2009), was established before the origin of jawed vertebrates, and is found in all vertebrate lineages where it mediates a variety of functions (Panksepp, 1998; Khan et al., 1999; Dreborg et al., 2008; Sundström et al., 2010). Pain and reward/pleasure affect are two prominent roles in mammals (Panksepp, 1998; Taylor et al., 2000; Dreborg et al., 2008). Whether these affective functions are shared in other lineages is poorly studied, but available data suggest that both roles exist in birds (Panksepp et al., 1980; Riters et al., 2014), pain/nociception roles exist in amphibians (Stevens, 2004), and both roles exist in teleosts (Sneddon, 2004; Lau et al., 2006; Bretaud et al., 2007) (but see Grant, 1992).

In addition to neurochemicals, brain regions involved in *M. ochrogaster* pair bonding present a high degree of functional homology across vertebrates (prefrontal cortex notwithstanding, because it's homologs are currently unknown) (Rink and Wullimann, 2001, 2002, Portavella et al., 2002; Wullimann and Mueller, 2004; Broglio et al., 2005; Northcutt, 2006, 2008; Bruce and Bradford, 2009; Nieuwenhuys, 2009). Finally, it has been most recently discovered that protein and gene expression patterns of socially-paramount neurochemicals across key brain regions that regulate reward and social behaviour are strikingly similar across vertebrates (O'Connell and Hofmann, 2011,

2012). This highly conserved social decision making (SDM) neural network, comprised of two sub-circuitries (the mesolimbic dopaminergic system (MDS) (Wise, 2002), and social behaviour network (SBN) (Newman, 1999; Goodson, 2005)), were already established in ancestral osteichthyes ~450 MYA (O'Connell and Hoffmann, 2011, 2012). Notably, nonapeptide and dopamine systems, as well as brain regions involved in *M. ochrogaster* pair bonding (PFC notwithstanding once more) are constituents of the vertebrate SDM network (**Figure 3.1**).

Given that the neurochemical and neuroanatomical components that underpin pair bonding in *M. ochrogaster* originated in early vertebrates and have remained structurally and functionally conserved, it is conceivable that in at least selective cases, they may have been repeatedly co-opted and similarly assembled into a converged regulatory neural network during independent transitions to pair bonding within the sub-phylum. Indeed, nonapeptide and DA systems appear to regulate pair bonding in other species that span several lineages, and appear to do so through targeting similar brain regions. In male and female marmosets, *Callithrix penicillata*, OT promotes while an OTR antagonist reduces affiliation during cohabitation with a prospective partner (Smith et al., 2010). Similarly, in male and female tamarins, *Saquinus Oedipus*, urinary OT increases with intra-pair affiliation (Snowdon et al., 2010). In zebra finches*, T. guttata,* both i.c.v. and peripheral OTR antagonist administration impairs pair bonding behaviors, including latency to pair, and pairing stability (Pedersen and Tomaszycki, 2012; Klatt and Goodson, 2013). In male cichlids, *Amatitlania nigrofasciata*, a IT/AVT antagonist cocktail inhibits affiliation with prospective partner and aggression towards non-partners (Oldfield and Hofmann, 2011). However nonapeptides do not appear to be involved in male *A. nigrofasciata* pair bond maintenance (Oldfield and Hofmann, 2011; O'Connell et al., 2012). Pair bonding pine voles, *M. pinetorum,* exhibit higher NAcc OTR expression (Insel and Shapiro, 1992) and VP V1aR densities (Insel et al., 1994) than do non-pairing montane voles, *M. montanus*. In five species of zebra finches (f: Estrildidae), LS V1aR density predicts species-typical social group sizes (Goodson et al., 2006). Similarly, in seven species of butterflyfishes (f: Chaetodontidae) AVT-ir neuron fibre varicosity density within the lateral septum-like region (the ventral and lateral parts of the ventral telencephalon, Vv/Vl) predicts species-typical pairing from non-pairing sociality (Dewan et al., 2011). Finally, in zebra finches, *T. guttata*, during pair bond formation and in established pairs, DA neurons expressing immediate early gene Fos (a marker of neuron activity) in the VTA is heightened (Goodson et al., 2009; Banerjee et al., 2013), and pair bonded birds exhibit higher levels of DA in the ventral medial striatum (the super-structure of the NAcc) than unpaired birds (Banerjee et al., 2013). However, since comprehensive examination of both the functional involvement of nonapeptide, dopaminergic, and opioid systems, and their respective targeting sites is thus far limited to *M. ochrogaster,* it is currently not possible to confidently discern the extent to which pair bonding regulatory neural networks may have converged across vertebrates.

The aim of this chapter was to explore the functional involvement of IT, V1a, DA, and MO receptors in pair bonding in a teleost, *Chaetodon lunulatus,* as well as establishing the specific brain region(s) (anatomical substrate(s)) upon which each receptor type operates*.* The specific study species, *C. lunulatus*, was selected due to its' strong pair bonding phenotype and tractability for both experimental and comparative research (Chapter 2). This study involved two separate components. Firstly, pharmacological tests were conducted to establish the functional involvement of ITR, V1aR, D1R, and MOR receptors. Males were adminstered receptor antagonist to test for changes in base-line selective affiliation with their established partner. Nowicki et al. (Chapter 2) previously showed that selective partner affiliation is a hallmark of wild pair-forming butterflyfishes that can be evoked in *C. lunulatus* under the classic laboratory two-choice proximity (or partner preference) paradigm used to study pair bonding in *M. ochrogaster* and other species (Williams et al., 1992; Adkins-Regan, 1998, 2016) enabling similar manipulation and testing of pairing in this species. Secondly, comparative analyses of receptor gene expression in eight distinct brain regions (**Table 3.1**) were undertaken to explicitly contrast pair bonded and solitary conspecifics, attempting to establish the specific brain regions that operate as neurochemical substrates for each of the four receptors. Importantly, these regions include the putative ancestral homologs of those involved in *M. ochrogaster* pair bonding (**Table 3.1**).

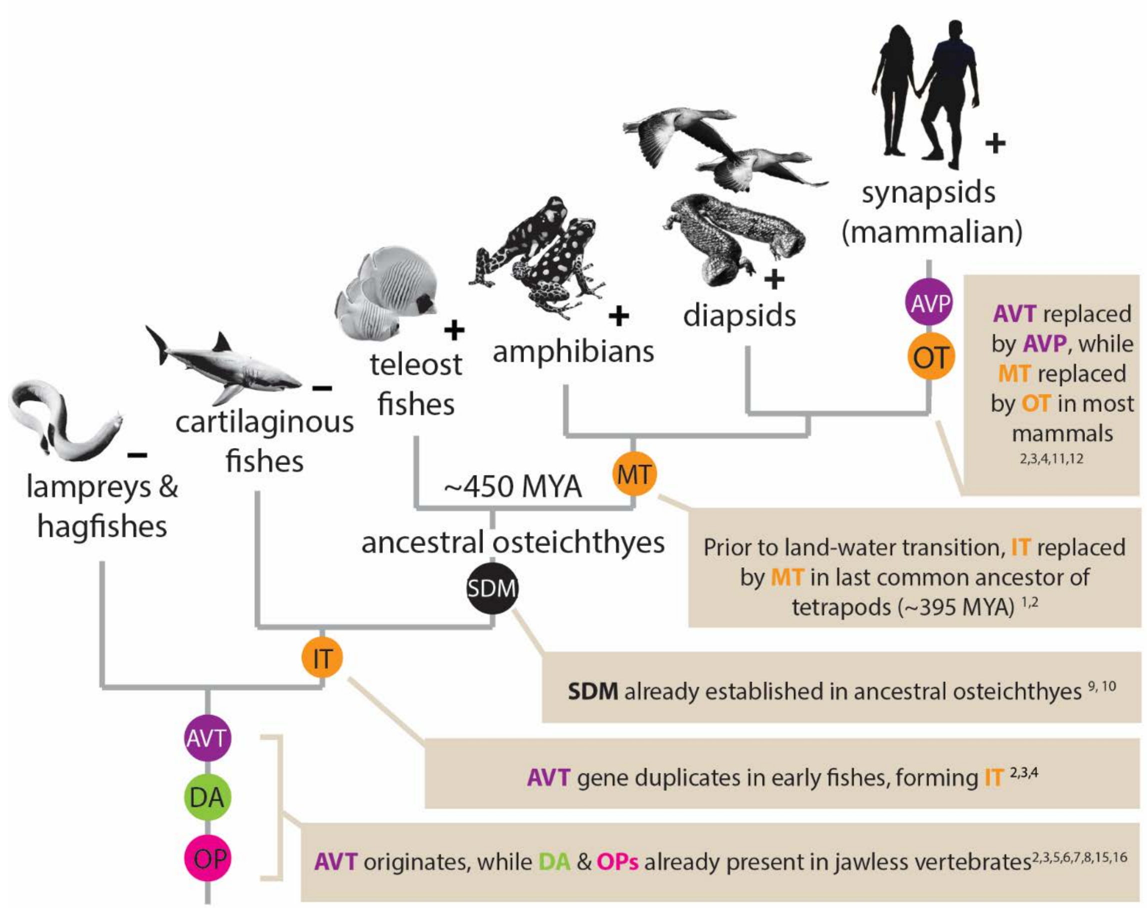


Figure 3.1. The neurochemical and -anatomical substrates of *M. ochrogaster* pair bonding pre-date the split between ray- and lobe-finned fishes, and have since remained highly conserved. Hence, the convergence of vertebrate pair bonding may have relied on repeatedly co-opting these homologies that were already established in a common ancestor ~ 450 MYA. The distribution of vertebrate pair bonding is indicated by its presence (+) or absence (-) across major groups. The evolutionary history of neurochemical systems (colored circles) and brain regions comprising the SDM (black circle) is shown.

Abbreviations: AVT = arginine vasotocin; DA = dopamine; OP = opioid; IT = isotocin; SDM =social decision making network; MT = mesotocin; AVP = arginine vasopressin; OT = oxytocin; SDM = social decision making network. Cladogram re-drawn from Butler and Hodos, 2005.

References for neural components: [1]Shubin, 2008; [2]Goodson, 2008; [3]Archer and Chauvet, 1995; [4]Archer et al., 1995; [5]Callier et al., 2003; [6]Yamamoto and Vernier, 2011; [7]Khan et al., 1999; [8]Le Merrer et al., 2009; [9]O'Connell and Hofmann, 2011, [10]O'Connell and Hofmann, 2012; [11]Moore, 1992; [12]Moore and Lowry, 1998; [15]Dreborg et al., 2008; [16]Sundström et al., 2010.

Sources for distribution of pair bonding among groups: *Mammals:* Porton, 1983*;* McWilliam, 1987*;* Heller et al., 1993*;* Crooks and VanVuren, 1996*;* Fuentes, 2000*;* Ralls et al., 2007*;* Munshi-South, 2008*;* Glenn et al., 2009*;* Jácomo et al., 2009*;* Wright et al., 2010*;* Marino et al., 2012*;* Seidler and Gese, 2012*;* Opie et al., 2013*;* Lukas and Clutton-Brock, 2013*;* Poessel and Gese, 2013*;* Jordan et al., 2014*;* Friesen et al., 2015*;* Funakoshi et al., 2015. *Birds: Lack*, 1968; Griffith et al., 2002; Cockburn, 2006. *Reptiles:* Bull, 2000*;* Chapple, 2003. *Amphibians:* Caldwell, 1997*;* Gillette et al., 2000*;* IUCN Red

List, 2007; Brown et al., 2008; Brown et al., 2010. *Fishes:* Pratt et al., 2001; Whiteman and Côté, 2004; Froese and Pauly, 2012; Brandl and Bellwood, 2014.

Table 3.1. Teleost brain regions examined in current study, and their putative mammalian homologs.

Teleost brain region	Putative mammalian homolog***
1. Medial part of the dorsal telencephalon (Dm)	Basolateral amygdala (blAMY)[1,2,3]
2. Dorsal part of the ventral telencephalon (Vd)	Nucleus accumbens(NAcc)[3, 11] and striatum (Str/CP)[3,12,13,14]
3. Lateral part of the dorsal telencephalon (Dl)	Hippocampus (HIP)[1,2,3]
4. Ventral and lateral parts of the ventral telencephalon (Vv/vl)	Septum, lateral septum (LS)[3,2,4,5,6]
5. Supracommissural nucleus of the ventral telencephalon (Vs)	Extended amygdala (medial amygdala/bed nucleus of stria terminalis (meAMY/BNST))[3,5]
6. Central nucleus of the ventral telencephalon (Vc)	Striatum (Str)[3,8]/ caudate putamen (CP)**
7. Preoptic area and (POA/PVN)	Preoptic area (POA)[3,7]
8. Periventricular nucleus of posterior tuberculum (TPp)	Ventral tegmental area (VTA)[3,9,10*]/ substantianigra pars compacta (SNc)[15]

References: [1]Portavella et al., 2002;[2]Northcutt, 2006;[3]O'Connell and Hofmann, 2011; [4]Wullimann and Muller; 2004; [5]Northcutt, 1995; [6]Bradford, 1995; [7]Moore and Lowry, 1998, [8]Wullimann and Rink, 2002; [9]Rink and Wullimann, 2001; [10]Luo et al., 2008; [11]O'Connell et al., 2011; [12]Sharma et al., 1989; [13]Batten et al., 1990; [14]Weld and Maler, 1992; [15]Fallon and Moore, 1978

Notes: *The teleost TPp has been suggested to be at least functionally equivalent (Rink and Wullimann, 2001) if not homologous (Lou et al., 2008) to the VTA/substantianigra pars compacta (SNc) (Fallon and Moore, 1978).

**In most mammals, the caudate putamen is a sub-structure of the striatum (O'Connell and Hofmann, 2011).

***While a tentative consensus for putative partial homologies between mammals and teleost brain regions has emerged, homologies should still be considered debatable (O'Connell et al. 2011; Goodson and Kingsbury, 2013).

****Brain regions involved in *M. ochrogaster* pair bonding that were not examined in the current study include the PFC and the VP (because their ancestral homologs are unknown (O'Connell and Hofmann, 2011)), and the anterior hypothalamus (teleost homologue = vTn) (O'Connell and Hofmann, 2011).

3.3 Methods

3.3.1 Study location

This study was conducted at Lizard Island, located in northern section of the Great Barrier Reef (GBR), Australia (14°40’08”S; 145°27’34”E). To explore social behavior independently of reproductive behavior, studies were undertaken during the winter months (May-July, 2013 and 2015), when spawning is expected to be at a seasonal low. Although the reproductive season of butterflyfish at Lizard Island is currently unknown, spawning by butterflyfishes is generally constrained at low temperatures (Yabuta and Berumen, 2014). The absence of reproductive activity was confirmed by the absence of yolked oocytes found among all females collected for the pharmacology experiment, except for one (see sex determination below). Since pair formation in butterflyfishes generally corresponds with reproductive maturation (e.g., Pratchett et al. 2006) only only individuals that were within 80% of the asymptotic size for the species, and therefore likely to be reproductively mature (Pratchett et al., 2006) were considered in this study.

3.3.2 Pharmacological tests of neurochemical receptors

Males were chosen as the focal sex, as per the majority of pharmacological studies on pair bonding (e.g., Winslow et al., 1993; Young et al., 1997, 1999; Liu et al., 2001; Lim and Young, 2004; Jarcho et al., 2011; O’Connell et al., 2012), to facilitate meangingful comparisons across studies. Pairs of *C. lunulatus* (n = 12 pairs from which females were used as the “non-partner” and n = 8 pairs/receptor antagonist treatment) were collected from the fringing reefs on the western side of Lizard Island using a barrier net. Pairs were identified as two individuals that displayed exclusive proximate swimming (within 1.5m distance) for at least 3 consecutive minutes throughout a 5-minute observation period, following Nowicki et al. (Chapter 2). Multiple but separated pairs were housed in a large flow-through aquaria (volume ~300 - >1,000 liters) lined with sand, and each pair’s compartment contained dead branching coral for habitat, as well as live coral (*Acropora intermedia)* for food. Individuals of each pair were sexed using gonad catheterization following Tricas (1989). Total length (TL) was then recorded for each individual. After experiments, pairs were returned to the reef site from which they were collected and fin clipped prior to release to avoid re-sampling of the same individuals.

3.3.2.1 Partner preference assay

To measure receptor involvement in selective affiliation with a partner, a testing tank (2.2m long X 1.2m wide X 0.5m deep; volume: 1,000 litres) was divided into three chambers using clear mesh netting, allowing for transmission of visual, olfactory, and auditory cues; which are involved in individual and conspecific recognition in butterflyfishes (Tricas et al., 2006; Boyle and Tricas, 2014; Tricas and Boyle, 2015). The central chamber was further divided into three fully accessible zones that were created using string that was suspended above the water surface: two outer testing zones that were adjacent to either the partner's chamber or the non-partner's chamber, and one central zone. The tank was lined with sand, and each chamber contained one live colony of *Acropora intermedia* (ca. ~1 cm diameter) for food, as well as similar sized dead corals for shelter. For the central compartment, both the food and shelter were placed in the middle (neutral) zone, such that feeding and habitat-associations did not influence assessments of partner preference. Selective affiliation with partner was tested by placing a male from a focal pair in the central chamber of the testing tank and its' corresponding partner in one of the outer chambers, and another female from a different pair in the opposite outer chamber. Intra- and extra-pair females were of similar size (< 2 mm difference in TL). Selective affiliation with partner and general social affiliation were quantified by recording the position of the focal male relative to the three distinct testing zones every 30 seconds for one hour (**Figure 3.2A**).

3.3.2.2 Pharmacology experiments

Pairs were placed in the central compartment of the experimental setup and allowed to habituate overnight. The following morning, partner and non-partner females were placed in their respective chambers, and all fishes were left to acclimate for 2 hrs (08:00-10:00). Following, baseline levels of selective partner affiliation and general social affiliation were established. Immediately thereafter, the focal male was removed and delivered either a saline control or receptor blockade treatment. The positions of intra- and extra-pair females were then switched and opaque barriers were placed between chambers, and the treatment male was re-introduced into the central zone of the center chamber to undergo drug activation (or in the case of saline a "sham" drug activation) in isolation for 30 min. Thereafter, the opaque barriers were removed, and tests of partner and social preference were repeated (**Figure 3.2 B**). All behavioral observations were conducted from behind mesh shade cloth to minimize observer interference.

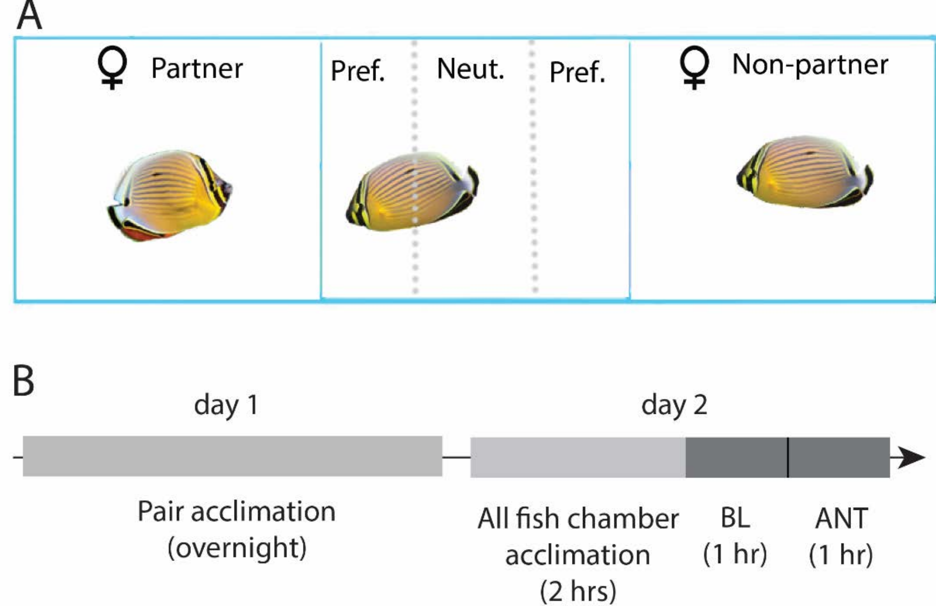


Figure 3.2. Partner preference paradigm used to examine response of male's selective affiliation with partner and general social affiliation to saline or receptor antagonist injection. (**A**) Schematic representation of testing tank. A tank was physically separated into three chambers using mesh material, allowing for transmission of social cues except tactile. The central chamber was further divided into three equally and fully accessible zones: one adjacent to female partner, one adjacent to female non-partner, and one central zone adjacent to no female. Behavior was recorded by an observer behind mesh cloth. (**B**) Schematic representation of protocol. On day 1, the focal pair acclimated to the test tank by residing in the central chamber overnight. The following morning (day 2), all test fish were placed in their respective chambers and acclimated for 2 hrs. Following, testing began by recorded the zone in which male was positioned every 30 seconds throughout a 1-hour observation period (base-line, BL). Following, males were removed and intra-dorsomuscularly administered either saline, ITR, V1aR, D1R, or MOR antagonist (ANT). The position of partner and non-partner females were then swapped, males were replaced in the choice arena, and the procedure repeated.

Receptor blockades or saline controls were delivered intramuscularly at a volume of 3.33µL solution/g wet body weight (w.b.w) using a 28.5G insulin syringe. I used the selective oxytocin receptor antagonist desGly-NH_2-d$(CH_2)_5$[D-Try^2,Thr^4]OVT (Manning et al., 2008); the selective µ-opioid receptor antagonist, CTAP (Sigma Aldrich); the selective dopamine-1 receptor class antagonist, SCH23390 (Sigma Aldrich); and selective arginine vasopressin V1a receptor antagonist, SR49059 (Sigma Aldrich) as receptor blockades and 1 X phosphate buffered saline (PBS) as a control. Although both D1 and D2 receptor classes are involved in mammalian pair bonding, the D1 class is implicated in pair bond maintenance in particular (Aragona et al., 2006). Since subjects were being tested for pair bond maintenance, I only examined the D1R receptor class. All drugs were delivered peripherally, based on the premise that they would sufficiently

pass the blood brain barrier (BBB), act on the brain; and therefore have centrally-mediated effects. This premise is empirically supported for CTAP and SCH23390, since both have been shown to penetrate the BBB in rodents (Abbruscato et al., 1997; Martel et al., 1996). BBB penetration of desGly-NH_2-d$(CH_2)_5$[D-Try^2,Thr^4]OVT remains untested, whereas one study shows that arginine vasopressin V1a receptor antagonist, SR49059 does not penetrate the blood brain barrier in rodents (Tribollet et al., 1999). However, given that fish blood-brain barriers may be more permeable than mammals (and birds) (Bernstein and Streicher, 1965; Olson et al., 1978), it is conceivable that when delivered peripherally at sufficient doses, passive transport of minute yet effective amounts might occur in fishes.

To establish the optimal receptor blockade dose, I started with effective drug type-specific doses established for other teleosts, where available (Johansson et al., 2005; Braida et al., 2012; O'Connell and Hofmann, 2012). If an effect was not found, a step-wise 10-fold increase in dose was trialed. In the main experiments, 0.5µg/g w.b.w desGly-NH_2-d$(CH_2)_5$[D-Try^2,Thr^4]OVT, 0.05µg/g w.b.w CTAP, 0.005µg/g w.b.w SCH23390, and 1.0^{-5}µg/g w.b.w SR49059 were used. During drug preparation, desGly-NH_2-d$(CH_2)_5$[D-Try^2,Thr^4]OVT was dissolved in 1 X PBS vehicle to a concentration of 0.15µg/uL and stored at 4˚C for up to 10 days. CTAP was dissolved in 1 X PBS vehicle at a concentration of 0.015µg/µL, from which multiple 350µL aliquots of solution were stored at -20˚C protected from light for up to 1 week, with each aliquot undergoing one freeze-thaw immediately before use. SCH23390 was dissolved in sterile deionized water vehicle at a concentration of 0.0015µg/µL from which multiple 350µL aliquots of solution were stored at -20˚C for up to one week, with each aliquot undergoing one free-thawing immediately before use. SR49059 was dissolved in dimethyl sulfoxide (DMSO) vehicle to a concentration of 0.677µg/uL and then diluted to 3.0^{-6} µg/µL with 1 X PBS vehicle from which multiple 350µL aliquots of solution were made and stored at -20˚C for up to two weeks, with each aliquot undergoing one freeze-thaw immediately before use.

3.3.2.3 Statistical analyses of pharmacology experiments

For each treatment, the proportion of time spent with partner versus non-partner females was compared between base-line and treatment phases using a non-parametric Wilcoxon signed-rank test, due to over-dispersion of residual data. The statistical analysis was conducted using SPSS software.

3.3.3 Neuro-receptor and -anatomical correlates of pair bonding

3.3.3.1 Animal collection and sexing

To compare receptor gene expression within brain regions between pair bonded and solitary individuals, I first collected solitary and paired individuals of *C. lunulatus* from fringing reefs around Lizard Island. The social system of individuals was recorded following 5-min observations prior to collecting fishes by spearing through the dorsal musculature. Individual fishes were immediately placed in an ice-water slurry for 5 min after which the brain was dissected (within 10 minutes of capture), embedded in optimal cutting compound (OCT), and frozen in liquid nitrogen for transportation to the laboratory where they were then transferred to -80 ˚C freezer until sectioning. In order to sex individuals, gonads were removed and fixed in formaldehyde-acetic acid-calcium chloride (FACC) for at least one week. Thereafter, gonads were dehydrated in a graded alcohol series, cleared in xylene, embedded in paraplast, sectioned transversely (7 µm thick), and stained with hematoxylin and eosin. Sections were examined under a compound microscope (400 X magnification) for the presence of sperm (male) or oocytes (female) (Pratchett et al., 2006).

3.3.3.2 Brain region extraction and measuring gene expression

Frozen brains were transversely sectioned on a cryostat at 110µm, thaw mounted onto Superfrost Plus slides (Fisher Scientific) and stored at -80˚C prior to brain region extraction (approx. one week). Brain regions, identified using a butterflyfish brain atlas (Bauchot et al., 1989; Dewan and Tricas, 2014), were manually extracted at -30˚C using a hand-held micro-punching device (50mm diameter; Stoelting, model # 57401) (**Figure 3.3**), incubated in RNAlater® at 4˚C over night, and then stored at -20˚C for up to one week. Brain region punching regime was standardized across individuals (see **Supplementary Table 3.1** for regime). Tissue punches were immediately transferred into a lysis buffer and homogenized by passing through a 21-gauge needle 15 times. Following, RNA was extracted using an E.Z.N.A® HP Total RNA kit (Omega, model # R6812-02) according to the manufacturer's instructions, and stored at -80˚C prior to cDNA synthesis. RNA was reverse transcribed into cDNA using Superscript III reverse transcriptase (Life Technologies) and gene-specific primers (see **Supplementary Table 3.2** for primer sequences). Residual primers and salts from reverse transcription were

removed using an E.Z.N.A ® Tissue DNA purification kit (Omega, product # D3396-02), and cDNA was stored at -20˚C for up to 10 days prior to qPCR.

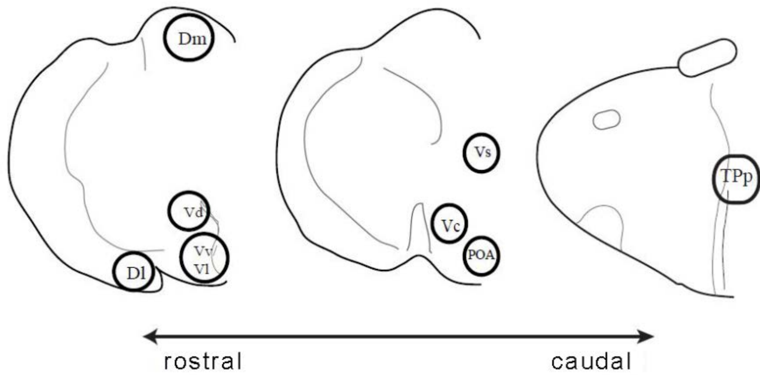


Figure 3.3. Transverse sections of oval butterflyfish brain are shown with circles identifying brain region micro-punches extracted for gene expression analysis.

The whole brain transcriptome of *C. lunulatus* was sequenced in order to use as a reference for designing species specific cloning primers for each gene of interest. One *C. lunulatus* brain was taken out of RNAlater, rinsed in 1X phosphate buffered saline (PBS) and placed immediately in Trizol (Life Technologies, Grand Island, NY) where RNA was extracted according to manufacturer instructions. Poly-adenylated RNA was isolated from each sample using the NEXTflex PolyA Bead kit (Bioo Scientific, Austin, TX, USA). Lack of contaminating ribosomal RNA was confirmed using the Agilent 2100 Bioanalyzer. A strand specific library was prepared using the dUTP NEXTflex RNAseq kit (Bioo Scientific), which includes a magnetic bead-based size selection of roughly 350 bp. The library was pooled in equimolar amounts with sample from an unrelated study after library quantification using both quantitative PCR with the KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA, USA) and the fluorometric Qubit dsDNA high sensitivity assay kit (Life Technologies), both according to manufacturer instructions. Libraries were sequenced on an Illumina HiSeq 2000 to obtain paired-end 100bp reads. I first corrected errors in the Illumina reads using Rcorrector (parameters: run_rcorrector.pl -k 31) and then applied quality and adaptor trimming using Trim Galore! (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/; parameters: trim_galore --paired --phred33 --length 36 -q 5 --stringency 5 --illumina -e 0.1). After filtering and trimming, a total of 64,795,096 paired reads remained for de novo assembly. I created a *C. lunulatus* de novo transcriptome assembly using Trinity (parameters: --seqType fq --SS_lib_type RF). The raw Trinity assembly produced 376,338 contigs (N50: 1148 bp).

Using the *C. lunulatus* transcriptome as a reference, species-specific primers were designed to clone target gene sequences. Cloned target sequences were examined to determine whether they contained an exon-exon boundary using *Danio rerio* and *Stegastes partitus* complete genomes as a reference. If target gene sequences did not

flank an exon boundary, then a second set of primers were designed to extend the obtained sequence towards the exon(s). Exon-containing sequences of ITR, V1aR, D1R, D2R and MOR genes were then used to design qPCR primers that flanked exon boundaries (18S ribosomal notwithstanding, because it does not contain an exon boundary) (see **Supplementary Table 3.2** for primer sequences). Prior to qPCR, primer sets and instrument cycling parameters were empirically optimized on standard curves using several metrics of quality control (i.e., assay amplification R^2 value of at least 0.95, assay slope of approximately -3.3, assay melting curve that only produced a single amplicon peak, no amplicon signal in the no template control (NTC) or nor reverse-transcription control (NRTC)). Quantitative PCR was then performed on each sample using a reaction mixture and qPCR cycling instrument (CFX380) that was recommended by the enzyme manufacturer (see **Supplementary Table 3.3** for parameters). Samples were run in technical triplicate on 384 well qPCR plates with standard curves in order to determine assay efficiency from the slope. Since assay efficiency was not the same across individual assays, averaged gene expression (C_t) values were standardized to assay efficiency prior to normalizing to 18S ribosomal RNA following methods of Simon (2003). Not all focal regions of each brain were measured for gene expression due to insufficient tissue available.

3.3.3.3 Statistical analysis of receptor and anatomical correlates of pair bonding

For each gene within each brain region, a 2-way ANOVA with social system and sex as fixed factors was used to determine whether gene expression varied independently or interactively among factor levels (α = 0.05). Prior to analysis, data was natural log transformed +1 to improve normality of residual variance. To account for multiple hypotheses testing of differential gene expression, for each brain region, a Bonferroni correction was applied during analysis. To identify differences between means, a Tukey's honest significant difference (HSD) test was applied *post hoc*. Statistical analysis was conducted using SPSS software.

3.4 Results

3.4.1 Effect of receptor antagonists on selective partner affiliation

The proportion of time that males spent affiliating with their female partner significantly declined from 0.90 ± .04 SE to 0.38 ± 0.13 SE (~ 58 %) following ITR (Z = -2.38, p = 0.02) and from 0.91 ± 0.03 SE to 0.31 ± 0.15 SE (~ 66 %) following V1aR (Z = -2.24, p = 0.03) antagonist administration. It also appeared to decline from 0.67 ± 0.07 SE to 0.38 ± 0.12 SE (~ 43 %) following D1R antagonist administration, however this was to a statistically insignificant extent (Z = -1.82, p = 0.07). It was not significantly affected by MOR antagonist (Z = - 1.22, p = 0.27) or saline (Z = - 0.56, p = 0.58) administration (**Figure 3.4**). The proportion of time that males spent affiliating with another female (irrespective of

whether she was his partner or the non-partner) was not affected by any receptor antagonist (ITR: Z = -0.94, p = 0.35; V1aR: Z = -0.59, p = 0.55; D1R: Z = -1.18, p = 0.24; MOR: Z = -1.22, p = 0.22) or by saline (Z = -0.28, p = 0.78) administration.

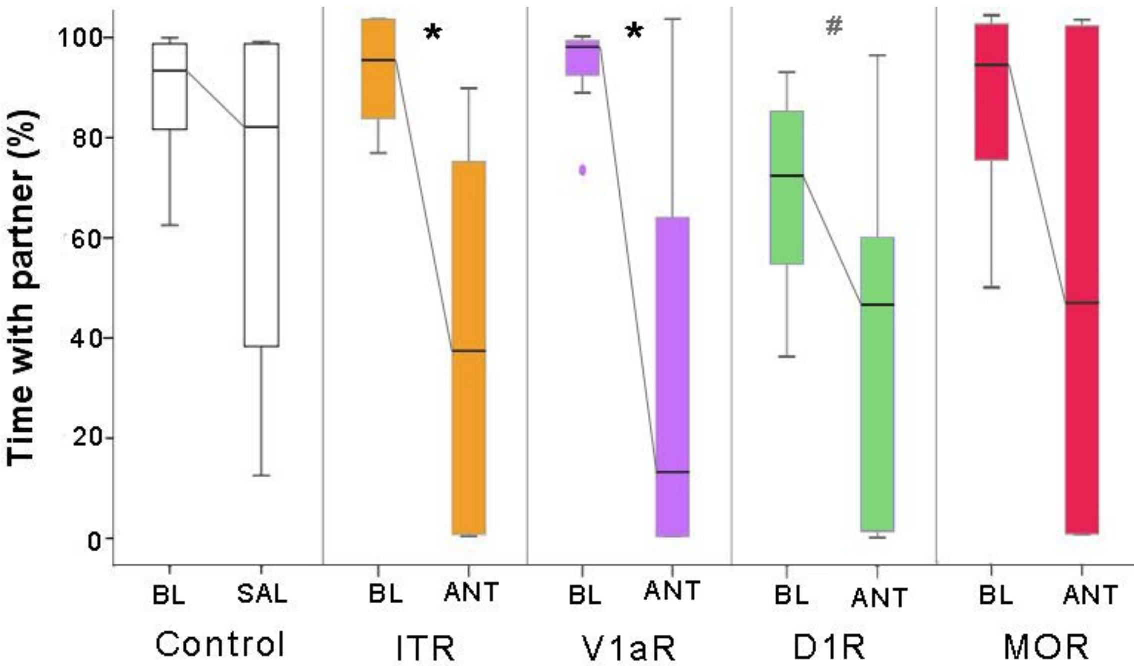


Figure 3.4. Response of male's (n = 8) selective affiliation with partner to saline or receptor antagonist injection. Boxplots show the percentage of time that males spent selectively affiliating with their partner prior to (base-line, BL) and following either saline (SAL) or antagonist (ANT) treatment. Boxes show first and third quartiles, black line in box represents median, and whiskers represent min. and max. values. * = statistically significant ($p < 0.05$), and # = appears different but to a statistically insignificant extent ($p = 0.07$), differences between treatment groups via Wilcoxon signed-rank test.

3.4.2 Neuro-receptor and -anatomical correlates of pair bonding

3.4.2.1 Nonapeptide receptors (ITR and V1aR)

Both ITR and V1aR expression within the Vv/vl differed interactively between sex and social system (**Figure 3.5 A, B**; **Supplementary Table 3.4**). In females, ITR and V1aR Vv/Vl gene expression was higher in pair bonding than solitary individuals ($F_{1,13}$ = 9.06, p = 0.01; $F_{1,13}$ = 9.18, p = 0.01, respectively); however, in males, there was no difference in either ITR or V1aR Vv/Vl gene expression between social systems ($F_{1,13}$ = .002, p = 1; $F_{1,13}$ = .036, p = 1, respectively). ITR and V1aR Vv/vl gene expression differed significantly between sexes ($p < 0.05$), with females having higher nonapeptide gene expression than males; and they also differed between social system ($p < 0.05$), with pair bonded individuals displaying higher nonapeptide gene expression than singletons. Gene expression of both ITR and V1aR did not differ between sex or social system interactively

or independently in any other brain region, namely in the Dl, Dm, POA, TPp, Vc, Vc, or Vs (**Supplementary Table 3.4**). V1aR gene expression was detected in all brain regions examined (ie., the Dl, Dm, POA, TPp, Vc, Vd, Vv/Vl, and Vs) and ITR gene expression was detected within all brain regions except for the Vd.

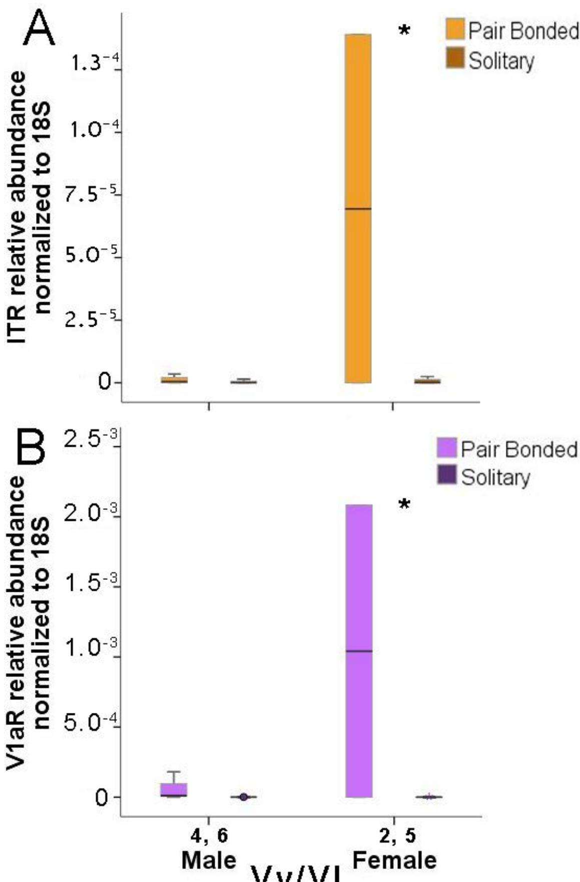


Figure 3.5. ITR (A) and V1aR (B) gene expression differences between sexes and social systems of *C. lunulatus* within the ventral and lateral parts of the ventral telencephalon (Vv/Vl). Boxes show the first and third quartiles, the black line in box represents median, and whiskers represent minimum and maximum value. Sample sizes are listed below each treatment group. Asterisks indicate statistically significant differences between treatment groups ($P < 0.05$) 2-way ANOVA and HSD Tukey Test.

3.4.2.2 Dopamine receptors (D1R and D2R)

For both dopamine receptor gene classes, gene expression did not vary between sex and social state within any brain region (**Supplementary Table 3.4**). Likewise, for both dopamine receptor gene classes, no difference in gene expression was found between sexes within any brain region (**Supplementary Table 3.4**). However, in several brain regions, gene expression of both dopamine receptor classes differed between social systems (p <0.05 for each region), with both male and female pair bonded individuals expressing less than their solitary counterparts in these areas: D1R: POA; D2R: Dm, Dl, Vs, POA, Vc, and TPp (**Figure 3.6A, B**; **Supplementary Table 3.4**). Dopamine receptor class expression was statistically similar between social systems in other brain regions: D1R: Dm, Dl, TPp, Vc, Vd, Vs, Vv/vl; D2R: Vd, Vv/vl (**Supplementary Table 3.4**) Although D1R receptor expression within the Vs appeared lower in pair bonded individuals than in solitary counterparts, this was to a statistically insignificant extent (p = 0.063). D1R and D2R gene expression was found in all brain regions examined (ie., the Dl, Dm, POA, TPp, Vc, Vd, Vv/Vl, and Vs).

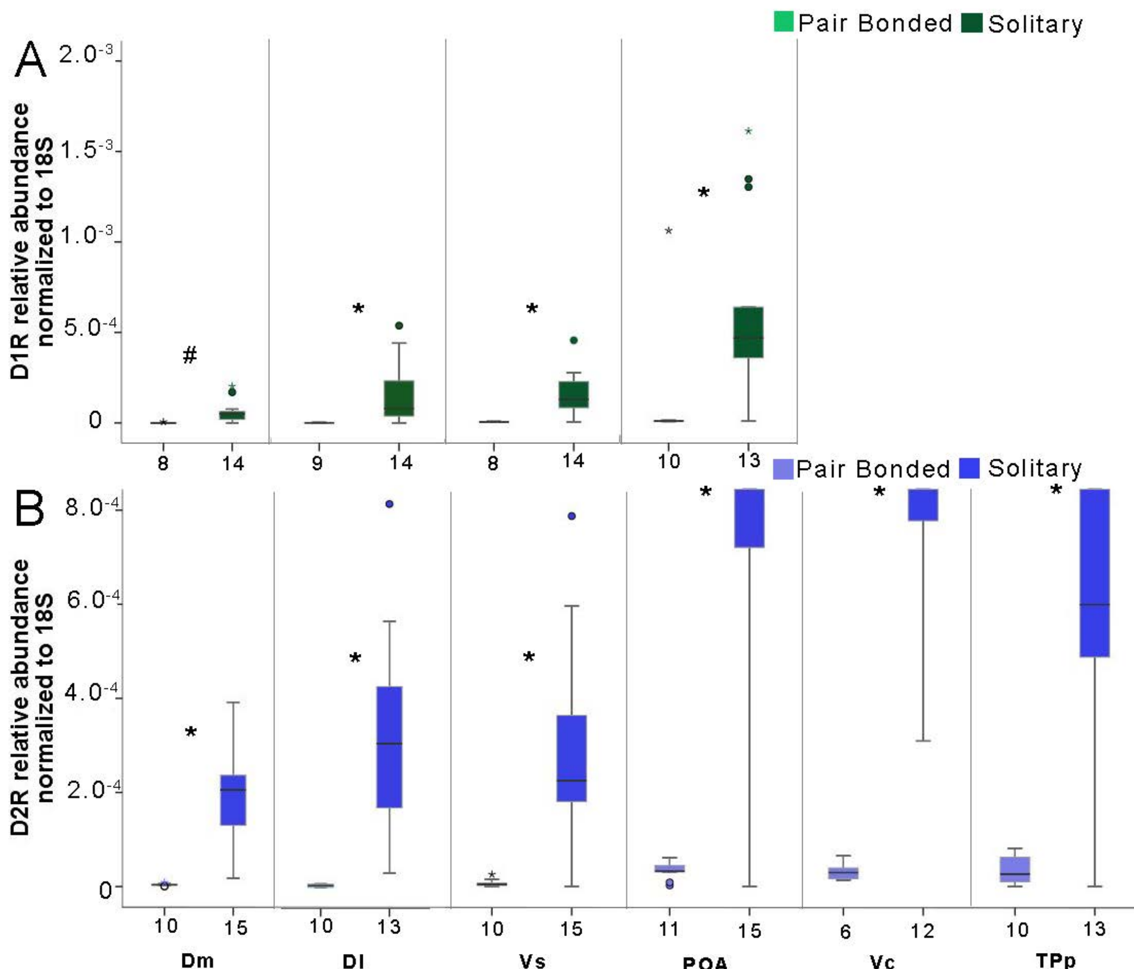


Figure 3.6. D1R (A) and D2R (B) gene expression differences between social systems of *C. lunulatus* within brain regions*.* Boxes show the first and third quartiles, the black line in box represents median, and whiskers represent minimum and maximum value. Sample sizes are listed below each treatment group. * = statistically significant ($p < 0.05$), and # = appearing but statistically insignificant (p= 0.053) differences between treatment groups (ANOVA). Abbreviations: Dm, medial part of the dorsal telencephalon; Dl, lateral part of the dorsal telencephalon; Vs, supracommissural nucleus of the ventral telencephalon; POA, preoptic area; Vc, central nucleus of the ventral telencephalon; TPp, periventricular nucleus of posterior tuberculum.

3.4.2.3 Mu-opioid receptor

Gene expression of MOR did not differ between sex and social system in any brain region, nor did it differ independently between sexes in any brain region (**Supplementary Table 3.4**). However, in two brain regions, MOR gene expression differed between social systems ($p < 0.05$ for each region), with both male and female pair bonded individuals expressing less than their solitary counterparts in the POA and TPp (**Figure 3.7A, B**; **Supplementary Table 3.4**). MOR receptor expression within the Dl, Vs, Vc, Dm, Vd, and Vv/Vl was statistically similar between social systems (**Supplementary Table 3.4**). MOR receptor expression within the Dl and Vs appeared

lower in pair bonded individuals than in solitary counterparts; however, this was to a statistically insignificant extent (p = 0.078 and 0.063, respectively). MOR gene expression was detected in all brain regions examined (ie., the Dl, Dm, POA, TPp, Vc, Vd, Vv/Vl, and Vs).

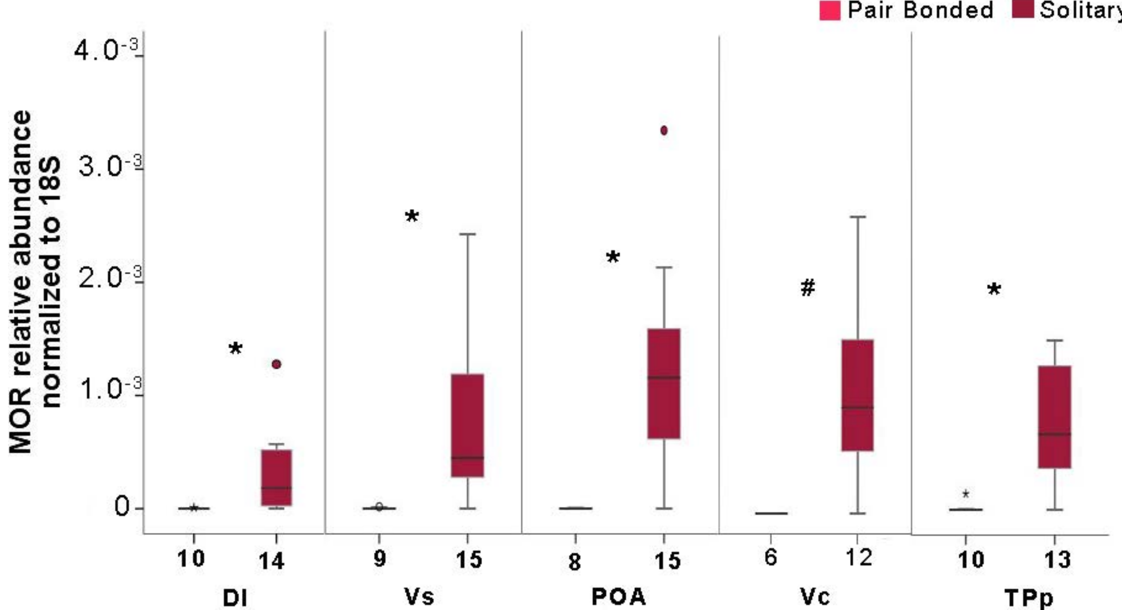


Figure 3.7. MOR gene expression differences between social systems of *C. lunulatus.* Boxes show the first and third quartiles, the black line in box represents median, and whiskers represent minimum and maximum value. Sample sizes are listed below each treatment group. * = statistically significant (p< 0.05), and # = appearing but statistically insignificant (p= 0.059) differences between treatment groups (ANOVA). Abbreviations: Dl, lateral part of the dorsal telencephalon; Vs, supracommissural nucleus of the ventral telencephalon; POA, preoptic area; Vc, central nucleus of the ventral telencephalon; TPp, periventricular nucleus of posterior

3.5 Discussion

3.5.1 Nonapeptide circuitries of pair bonding

3.5.1.1 In Chaetodon lunulatus

Administration of IT and V1a receptor antagonists strongly and significantly reduced the proportion of time that *C. lunulatus* males spent selectively affiliating with their established female partners (by 58 and 66 %, respectively); however, it had no effect on the percentage of time males spent affiliating with females in general. This provides functional evidence that both IT-ITR and AVT-V1aR nonapeptide systems promote pair bonding in males, and this is through promoting affiliation with their partner specifically rather than social affiliation in general. Results of the comparative analyses suggest that the brain region(s) on which IT-ITR or AVT-V1aR signaling act to exert this effect in males do not include any of the regions examined in the current study (i.e., the Dl, Dm, POA,

TPp, Vc, Vd, Vv/Vl, and Vs), since no dissimilarities of neither ITR nor V1aR gene expression within these regions were found between paired and solitary males. Although functional tests were only conducted on males, my comparative analyses revealed that in females, paired individuals displayed higher ITR and V1aR receptor expression within the Vv/Vl than solitary individuals, indicating that in females, ITR and V1aR signaling within the Vv/Vl might be important for mediating pair bonding. However, since sample size was small for both males and females, it is possible that for both sexes, there was insufficient power to detect true differences (or lack thereof) (Button et al., 2013). Hence, these results should be interpreted with caution. Few other studies have explored the involvement of nonapeptides in teleost pair bonding, and they have been mostly on males. In male cichlids, *A. nigrofasciata*, a general nonapeptide receptor antagonist inhibits affiliation with a prospective partner and aggression towards non-partners (Oldfield and Hofmann, 2011), indicating the involvement both systems in pair bond formation. However, nonapeptide signaling does not appear to be involved in pair bond maintenance in males of this species (Oldfield and Hofmann, 2011; O'Connell et al., 2012). In established pairs of *Neolamprologus pulcher* (but not of *Telmatochromis temporalis*) cichlids, whole brain gene expression of IT is positively correlated with partner affiliation (O'Connor et al., 2016). Additionally, *N. pulcher* displays higher whole brain gene expression IT than the less affiliative *Telmatochromis temporalis* (O'Connor et al., 2016). Similar to my study, Dewan et al. (2011) found in males of seven species of chaetodontids, Vv/Vl AVT-ir neuron fibre varicosity density predicts species-typical pairing from non-pairing sociality. Taken together, these studies indicate that while nonapeptides play a recurring role in promoting teleost pair bonding, this is species-, gender-, and context-specific.

What might be the precise functional role of IT-ITR and AVT-V1aR signaling (for females specifically within the Vv/Vl) in promoting *C. lunulatus* pair bonding? In teleosts, both AVT and IT mediate a wide range of behavioral domains that lack a universal valence and appear to be context specific (Godwin and Thompson, 2012). AVT regulation of social behavior has been studied extensively, albeit almost exclusively in males, where it has functionally been shown to promote spawning (Pickford and Strecker, 1977; Semsar et al., 2001; Carneiro et al., 2003), mate guarding or other forms of conspecific aggression/avoidance (Semsar et al., 2001; Semsar and Godwin, 2004; Thompson et al., 2004; Santangelo and Bass, 2006, 2010; Oldfield and Hofmann, 2011; Mendonca et al., 2013; Sakamoto et al., 2015), social communication (Bastian et al., 2001), social preference (Braida et al., 2012) and approach behavior (Filby et al., 2010). Few studies have functionally examined IT involvement in teleost social behavior and again, those which have are exclusive to males. Similar to AVT, these studies demonstrate an inconsistent effect of IT on approach and avoidance behaviors, including pair bond formation and maintenance (Oldfield and Hofmann, 2011; O'Connell et al., 2012), social preference (Braida et al., 2012), and social affiliation (Reddon et al., 2014). However, since neurochemical effects are often specific to the site(s) of action

(Veenema et al., 2010; Beery, 2015) and sites are not confirmed in these studies, it is difficult to know which, if any, of these roles generate insight into the current findings. The rich literature on rodents, however, shows that in pair bonding species, AVP within the lateral septum (LS, the mammalian homolog of Vv/Vl) is involved in both partner affiliation (Liu et al., 2001) and territoriality (Oldfield et al., 2015), perhaps reflecting its broader role in social recognition/memory (van Wimersma Greidanus and Maigret, 1996; Dantzer et al., 1987, 1988; Landgraf et al., 2003; Bielsky and Young, 2004; Bielsky et al., 2005). As with AVP, septal (including lateral septal) OT is essential for social recognition in rodents (Popik et al., 1992; Engelmann and Landgraf, 1994; Everts and Koolhaas, 1997; Bielsky et al., 2005). In pair bonding butterflyfish, both olfactory and visual cues are used for conspecific recognition (Boyle and Tricas, 2014), and are necessary to modulate relationships with partners, territorial intruders (Yabuta, 2002) and competitors for mates (Fricke, 1986). In teleosts, the ventral telencephalon is the major target of olfactory projections (Lopes Corrêa et al., 1998; Laberge and Hara, 2001; Hamdani and Doving, 2007), but not of optic neurons, nor is it innervated with the optic tectum (Schlussman et al., 1990; Yamane et al., 1996; Perez and Perez, 2003) the major brain region in which visual information is integrated and processed in vertebrates (Nevin et al., 2010). Taken together, I speculate that in *C. lunulatus,* V1aR and ITR activation (and for females, specifically within the Vv/Vl) might serve to enhance conspecific recognition via olfactory perception (Dewan et al., 2011). This is certainly an intriguing area of further inquiry.

3.5.1.2 Convergent evolution with birds and mammals

The teleost, mammalian, and avian lineages share striking similarities in nonapeptide-mediated pair bonding circuitry. I have shown here that, as in other teleosts (Dewan et al., 2011; Oldfield and Hofmann, 2011), AVT plays an important role in *C. lunulatus* pair bonding, and that its effects are likely exerted within the Vv/Vl through V1aR activation, mirroring the role of AVP in birds (Kingsbury and Goodson, 2014), and of AVP-V1aR binding within the LS (the mammalian homolog of Vv/Vl) in *M. ochrogaster* voles (Liu et al., 2001). Similarly, fMRI studies show that in humans, activation of the septum, which is rich in AVP binding sites (Loup et al., 1991), is associated with "obsessive love" (Acevedo et al., 2012). I have further discovered that, as in other teleosts (Oldfield and Hofmann, 2011; O'Connor et al., 2016), IT is important for *C. lunulatus* pair bonding, paralleling the functional involvement of OT in pair bonding *M. ochrogaster* rodents (Williams et al.,1994; Cho et al., 1999; Young et al., 2001), and in non-human primates (i.e., marmosets, *Callithrix penicillata*: Smith et al., 2010; tamarins, *Saquinus oedipus*: Snowdon et al., 2010).

As with AVP, OT activity is also implicated in human pair bonding: intranasal OT in men within romantic partnerships increases preferred interpersonal distance from non-partner females (Sheele et al., 2012, 2013), and plasma OT levels predict future

success rates in romantic relationships (Schneiderman et al., 2012). However, unlike the AVP/AVT system, the site(s) of OT action in humans and *M. ochrogaster* rodents (ie. the prefrontal cortex (PFC), and nucleus accumbens (NAcc) (the mammalian homologue of the Vd) (Young et al., 2001; Ross et al., 2009; Ophir et al., 2012)) are different than that of IT in teleosts (i.e., the lateral septum-like area). There are several potential explanations for this. First, since the evolutionary antecedent of the mammalian PFC is unclear (Reiner, 1986; Butler et al., 2011; but see Mueller et al., 2011) it couldn't be examined here. Secondly, and somewhat surprisingly, this study found no ITR expression in the NAcc/Vd at all, suggesting that this region is not important for pair bonding and social behavior in general in *C. lunulatus*. Alternatively, this could be an artifact of lack of tissue available for sampling. Given that ITR within the NAcc/Vd is expressed in teleosts (Huffman et al., 2012) and that the NAcc/Vd is considered a key node in the vertebrate SDM network, I suspect that the latter alternative is more plausible. Technical limitations notwithstanding, we might still expect anatomical targets of ITR/OTR-mediated pair bonding to be distinct between mammals and teleosts, due to differences in pre-existing neural circuitries that would have been available for co-option during their independent evolution. In mammals, a pre-established OT-mediated maternal bonding circuitry, in which the NAcc is a critical site of action (Olazabal and Young, 2006 a,b), is thought to have been recruited during the evolution of pair bonding (Lim and Young, 2006; Donaldson and Young, 2008; Numan, 2014; Rilling and Young, 2014). Since parental care did not precede the evolution of pair bonding in butterflyfishes (Fricke, 1986), this pre-existing circuitry would have been unavailable for co-option in these organisms.

3.5.2 Dopaminergic circuitries of pair bonding

3.5.2.1 In Chaetodon lunulatus

Administration of the D1 receptor antagonist appeared to reduce the time that *C. lunulatus* males spent selectively affiliating with their established female partners; however, this was to a statistically insignificant extent. Despite insignificant effects, the directionality of observed responses, along with results of my comparative analyses (see below) suggests that DA-D1R signaling may play a role in promoting pair bonding in *C. lunulatus*. Insignificant differences in pharmacological studies may have resulted due to suboptimal i) treatment dosage, treatment activation time, and/or testing paradigm. Additionally, it is possible that non-significant findings could be explained by a relatively small sample size leading to lack of sufficient power. Endogenous dopamine has been shown to increase with shoaling behavior (Buske and Gerlai, 2012) and with approach towards images of conspecifics (Saif et al., 2013) in gregarious zebrafish, indicating dopamine's broader involvement in teleost social affiliation.

An essential component of pair bonding is the reinforcement of partner affiliation (Freeman and Young, 2013), which relies on individuals to perceive their partners as rewarding (i.e., approach eliciting) through heightened "salience" (Aragona et al., 2006, Berridge and Robinson, 2003; Wise, 2004). In pair bonding prairie voles, conspecific affiliation is not naturally rewarding, so does not facilitate pair bonding independently (Freeman and Young, 2013). However, affiliation coupled with natural reward (specifically mating), is reinforcing and thus promotes pair bonding (Everitt, 1990). Therefore, mammalian pair bond formation is viewed to depend on conditioned reward learning, whereby individuals learn to associate their partner (conditioned stimulus) with mating (natural reward/unconditioned stimulus) (Robbins and Everitt, 1996; Wise, 1996; Ikemoto and Panksepp, 1999; Aragona et al., 2003). The associative reward learning involved in this conditioned partner preference (CPP) is dependent upon dopamine acting upon nodes of the mesolimbic reward system--a neural network where the salience of environmental stimuli is evaluated (Young et al., 2001; Wise, 2004; Young and Wang, 2004; Freeman and Young, 2013). Similar to pair bonding prairie voles, *in situ* partner removal experiments on *C. lunulatus* show that widowed males and females initially act agonistically when approached by opposite sexed conspecifics within their territory. However, persistent "stalking" by the intruder towards the widowed individual while foraging accompanies the development of a new pair bond (Chapter 4). Hence, *C. lunulatus* pair bonding might also rely on the learned association between partner (conditioned stimulus) and food (natural reward/unconditioned stimulus), and this associative learning might also be underpinned by dopamine acting upon reward circuitry. In support of this idea, in teleosts, both DA-D1R and -D2R binding are critical for psychostimulant/food reward learning (Mattioli et al., 1995; Lau et al., 2006; Bretaud et al., 2007; Darland et al., 2012; Vindas et al., 2014; Messias et al., 2016) and a network structured very similarly to the amniote mesolimbic reward system has been identified (O'Connell and Hofmann, 2011; O'Connell et al., 2011). Importantly, almost all of the brain regions that were associated with DA-mediated pair bonding in this study are nodes of this putative teleost mesolimbic reward system (POA notwithstanding).

My comparative results revealed that in both male and female *C. lunulatus,* D2R gene expression differed in the TPp and Vc between pair bonded and solitary individuals. This suggests that DA-D2R signaling within these regions may be important for pair bonding in both sexes of this species. The mammalian homologs of these brain regions, namely the ventral tegmental area (VTA) and striatum (STR), comprise the central ascending dopaminergic innervation pathway in the mesolimbic reward system (O’Connell and Hofmann, 2011; O'Connell et al., 2011). In mammals, this pathway appears to have been co-opted during the evolution of pair bonding in order to mediate partner reward learning (Gingrich et al., 2000; Freeman and Young, 2013). In teleosts, the TPp seems to have the densest cluster of DA-synthesizing cell bodies in the brain (O'Connell et al., 2011) and is considered the dopaminergic system ascending to the

striatum (Rink and Wullimann, 2002). Furthermore, DA-synthesizing neurons within the TPp are necessary for conditioned learning of place preference (Bretaud et al., 2007; Facciolo et al., 2012). Given the aforementioned homologies and functional similarities, I tentatively hypothesize that DA ascending from the TPp and binding to D2Rs within the striatal Vc might function to mediate partner--consumatory reward learning in a similar manner to the VTA-NAcc complex in mammals (Aitken et al., 2015). Yet the hypothesis that the TPp is a major source of DA in *C. lunulatus* pair bonding does not explain why it appears to be a potential target of DA action in this species? Perhaps the TPp is both a source and a site of action in DA-mediation of pair bonding in *C. lunulatus.* This possibility might also apply for mammalian counterparts, because the VTA-mPFC complex within the mesocorticolimbic pathway is reciprocally innervated (Swanson, 1982; Carr and Sesack, 2000; McFarland and Kalivas, 2001), and DA-synthesizing neurons within the VTA display a high density of dendrite D2 receptors (Callier et al., 2003).

My comparative results revealed several other potential sites of DA action that are shared by D1R and D2R targeting. This is consistent with the ideas that D1R modulates D2R mediated events (Paul et al., 1992) and that D1- and D2R subtypes function complementarily to mediate pair bonding behavior (Aragona et al., 2006). Three of these implicated brain regions, the Dm, Dl, and Vs, belong to the putative teleost mesolimbic reward system, and mediate emotional learning/memory (Portavella et al., 2002), relational/spatial/temporal memory (Portavella et al., 2002), and aggression/spawning (O'Connell and Hofmann, 2011), respectively. The final brain region implicated, the POA, is a node of the conserved social decision making neural network, where it mediates several social domains across vertebrates, including sexual activity and male aggression (Satou et al., 1984; Wang et al., 1997; Gammie and Nelson, 2000; Wong, 2000; Curtis and Wang, 2003; O'Connell and Hofmann, 2011). In voles, in particular, the mPOA appears critical for several pair bonding behaviors, including pair bond formation (Cushing et al., 2003), mating (Curtis and Wang, 2003), mate guarding, and territorial defense (Wang et al., 1997; Gammie and Nelson, 2000; Curtis et al., 2006). mPOA-mediated pair bond formation and mating in particular are believed to be attributed to dopamine (Curtis et al., 2006).

Hence, I propose that in *C. lunulatus*, D1 and D2 receptors might act synergistically within the Dm, Dl, Vs, and POA to mediate emotional, spatial/temporal, and sexual/mate-guarding mnemonic events involved in partner reward learning. Of final note, dopamine receptor expression within these brain regions was relatively *lower* in pair bonded fish than in solitary counterparts. This is somewhat contradictory, because since I hypothesize that DA binding promotes partner reward learning, signaling is expected to be relatively *higher* in paired fish. I offer two potential explanations for this. First, reduced DAR gene expression might reflect reduced receptor expression, which might act as a compensatory mechanism for heightened DA release

(Fauchey et al., 2000). Secondly, while gene expression often increases with the activity or abundance of protein products, it has also been shown to exhibit an inverse relationship (Vogel and Marcotte, 2012), as has been previously shown in pair bonding *M. ochrogaster* (Okhovat et al., 2015). Therefore, it is possible that relatively lower DAR gene expression (and MOR gene expression, see below) reflects relatively higher levels of receptor abundance or activation in association with *C. lunulatus* pair bonding.

3.5.2.2 Convergent evolution with birds and mammals

The teleost, bird, and mammalian lineages share some prominent similarities in dopamine-mediated pair bonding circuitry. My comparative results suggest that dopamine neurotransmission within the mesolimbic reward network is important for pair bonding in *C. lunulatus*, as appears to be the case in the zebra finch *T. guttata* (Goodson et al., 2009, Alger et al., 2011; Banerjee et al., 2013; Prior and Soma, 2015), and in *M. ochrogaster* rodents (Gingrich et al., 2000; Aragona et al., 2003, 2006). A notable brain region of this network that appears to be targeted by DA in all three taxa is the striatal Vc/ striatal NAcc (Aragona et al., 2003, 2006; Alger et al., 2011; Banerjee et al., 2013; current study). In addition, DA appears to act within the TPp (mammalian and avian VTA) and the POA in both *C. lunulatus* and *T. guttata* (Goodson et al., 2009; Alter et al., 2011; current study), but whether it targets these regions in mammals remains untested. Finally, my study further implicated that DA-D1R- and -D2R signaling within the Dm, Dl, and Vs might also regulate *C. lunulatus* pair bonding, but their involvement within homologous regions (i.e., the blAMY, HIP, and meAMY/BNST, respectively) remain untested in other taxa. Interestingly, however, a growing body of research implicates that DA targets similar regions of the mesolimbic reward system to regulate partner affiliation in humans (Fisher, 1998; Acevedo and Aron, 2014). For example, functional magnetic resonance imaging (fMRI) shows that striatal regions, as well as the VTA, AMY, and the HIP, which are rich in dopamine activity (Callier et al., 2003), are activated differently when participants view images of those with whom they're in an intense romantic or long-term, deeply-loving relationship; than when viewing pictures of other familiar individuals (Bartels and Zeki, 2000, 2004; Aron et al., 2005).

3.5.3 Mu-opioid receptor circuitry of pair bonding

3.5.3.1 In Chaetodon lunulatus

I found no functional evidence for MOR involvement in male *C. lunulatus* pair bonding, as blocking the MOR did not affect selective affiliation with female partner. This finding might be because MOR is not involved in male *C. lunulatus* pair bonding. Alternatively, MOR might be involved, but was undetected in my functional study due to suboptimal

treatment dosage, treatment activation time, and/or testing paradigm. Additionally, it is possible that non-significant findings could be explained by a relatively small sample size leading to lack of sufficient power. Given that in both sexes of *C. lunulatus* I found comparative evidence for MOR involvement in pair bonding (see below), I suspect that one of the latter reasons are more plausible.

Specifically, MOR gene expression varied in relation to pairing sociality within the POA and several nodes of the mesolimbic reward system: the Dl, Vs and TPp. Similarly, while MOR gene expression within the striatal Vc appeared to differ in relation to pairing sociality, this was to a statistically insignificant extent. In teleosts, the POA mediates social and feeding behavior (O'Connell and Hofmann, 2011). It is well established for mammals, that MOR plays an essential role in mediating the reinforcing effects of natural rewards (e.g., food, water, sex, social affiliation) and of psychostimulant rewards by eliciting motivational and pleasurable hedonic responses to these stimuli (van Ree and de Wied, 1980; Panksepp et al., 1978; Shippenberg et al., 1987; Hubner and Koob, 1990; Hiroi and White, 1993; Vanderschuren et al., 1995; Olmstead and Franklin 1997; Corrigall et al., 2000; Pecina and Berridge, 2000; Van Ree et al., 2000; Skoubis and Maidment, 2003; Fields, 2007; Soderman and Unterwald, 2008; Le Merrer et al., 2009). Preliminary investigations suggest that opioid and/or MOR action within mesolimbic reward system also mediates reward processing in teleosts (Lau et al., 2006; Bretaud et al., 2007).

In prairie voles in particular, the mu-opioid system plays a critical role in facilitating pair bond formation (Burkett et al., 2011, Resendez and Aragona, 2012; Resendez et al., 2013). Its effects are exerted within striatal regions of the brain, including the dorsal striatum, dorsomedial NAcc shell, and caudate putamen (Burkett et al., 2011; Resendez and Aragona, 2012; Resendez et al., 2013), where dorsal striatum MORs are believed to facilitate pair bond formation by promoting mating during CPP, and dorsomedial MORs are believed to do so by modulating the positive hedonics of mating during CPP (Resendez et al., 2013). (See section on dopamine for description of CPP cognitive process.) In teleosts, food is a natural reward whose reinforcing properties are modulated by the opioid system (Lau et al., 2006). In *C. lunulatus,* exclusive pair-wise feeding strongly coincides with pair bond formation and maintenance (Chapter 4). Hence, I hypothesize that OP-MOR binding within the POA and nodes of the mesolimbic reward system (i.e., the Vc, Dl, Vs, and TPp) promotes pair bonding in *C. lunulatus* by modulating the positive hedonics of natural consumatory reward during CPP learning. In further support of this idea, my comparative results revealed that the opioid and the dopaminergic systems appear to target several of the same nodes of the mesolimbic reward system (i.e., the Vc, Dl, Vs, and TPp), indicating that they might converge on these regions in order to underpin the learned association between consumatory reward affect and one's partner, respectfully, during the CPP process. Experimental research is needed to empirically test this hypothesis.

3.5.3.2 Convergent evolution with mammals

To date, potential involvement the opioid-mu-opioid system in pair bonding has only been examined in two species, *C. lunulatus* butterflyfishes (current study) and *M. ochrogaster* voles (Burkett et al., 2011; Resendez and Aragona, 2012; Resendez et al., 2012, 2013, 2016). In both organisms, it appears to play an important role, and effects seem to be exerted by acting upon nodes of the mesolimbic reward network. Specifically, I found comparative evidence that the striatal Vc -MORs are important in *C. lunulatus*, mirroring the role of striatal region (the NAcc and dorsal striatum) MORs in *M. ochrogaster* (Resendez et al., 2012, 2013). While several other nodes of the mesolimbic reward system (i.e., the Dl, Vs, TPp) and the POA were implicated in *C. lunulatus* pair bonding, the involvement of their homologs (i.e., the meAMY, LS, VTA and POA, respectively) in other taxa is yet to be explored functionally, so cannot be compared here. Interestingly, however, emerging evidence suggests that OP-MOR activity is important for pair bonding in humans as well, where here too its function is believed to be eliciting motivation and positive hedonics in response to romantic affiliation (Georgiadis et al., 2012; Troisi et al., 2011; Hsu et al., 2013). Moreover, the implicated brain regions involved share some similarities with those implicated in *C. lunulatus* and established in *M. ochrogaster*. Specifically, fMRI studies suggest that in humans, motivational aspects of partner preference formation are regulated by the dorsal striatum (Resendez et al., 2013), which is rich in MORs (Inagaki et al., 2015). Whereas, the positive hedonics of "romantic love" are associated with the AMY, septal fornix, and VTA (Bartels and Zeki, 2000; Aron et al., 2005)—all of which are also rich in MORs (Pfeiffer et al., 1982; Maurer et al., 1983).

3.5.4 Working model for the neural network of pair bonding in fishes

By synthesizing my current findings with available information on teleost neurochemical synthesis and projection pathways, and functional insight from pair bonding *M. ochrogaster* counterparts, we can now begin to assemble a working model for how isotocin, arginine vasotocin, dopamine, and opioid systems might interplay to comprise a broader neural network of *C. lunulatus* pair bonding (as illustrated in **Figure 3.8**). It is important to emphasize from the very onset that the only component of this working neural network model that derived from my findings is the involvement of IT-ITR, AVT-V1aR DA-DR, and OP-MOR signaling within brain regions (olfactory bulb notwithstanding), and that the remainder of this model is purely speculation.

I tentatively hypothesize that pair bonding in *C. lunulatus* relies on conditioned partner preference (CPP), as in the mammalian model, *M. ochrogaster* (Freeman and Young, 2013). Several lines of behavioral evidence support this hypothesis. Field observations reveal that solitary *C. lunulatus* do not find prospective partners naturally rewarding (i.e., they respond to prospective partners by agonism rather than approach).

Only after continued and exclusive cohabitation involving pair-wise foraging, is a pair bond developed (Chapter 4). After development, pair bonds are enduring, and are characterized by selective affiliation and feeding with partner, and selective agonism towards non-partners (Chapter 2). I propose that during CPP in *C. lunulatus*, individuals form a learned association between natural reward (food, unconditioned stimulus) and their new partner (conditioned stimulus) resulting in the new partner to take on rewarding properties, and thus reinforce selective approach behavior (Young and Wang, 2004). In my working model for this process, feeding activates the TPp (VTA), concurrently triggering OP-MOR and DA-D2R activity within the mesocorticolimbic reward system, which converges in the Vc (striatum), Dl (HIP), Vs (meAMY, BNST), TPp (VTA), and POA in particular, thereby modulating consumatory reward affect and reward learning/salience of partner-associated cues. In this pathway, the major source of DA projection to at least the Vc (striatum) is most likely the TPp (VTA) (Callier et al., 2003; O'Connell et al., 2012), while OP is most likely to originate from the hypothalamic nucleus lateralis tuberis (Vallarino et al., 2012). Meanwhile, olfactory cues from the partner are transmitted from the olfactory bulb (OB), ultimately reaching the Vv/Vl (LS), where IT and AVT nonapeptide activity converges to promote olfactory learning in females. The source of nonapeptide release originates from cell bodies within the POA (Van den Dungen et al., 1982; Batten et al., 1990; Holmqvist and Ekström, 1991). After pair bond formation, concordant D1R and D2R activity within the mesocorticolimbic reward system and POA modulates pair bond maintenance by mediating aggression towards non-partner conspecifics (Resendez and Aragona, 2013). Also involved in this neural network would be higher-order motor circuits that underpin the behavioral outcome of approach and affiliation towards partner, and aversion towards non-partners (not studied nor illustrated here) (Grillner et al., 2013). This proposed working model is speculative, and requires empirical testing (see below).

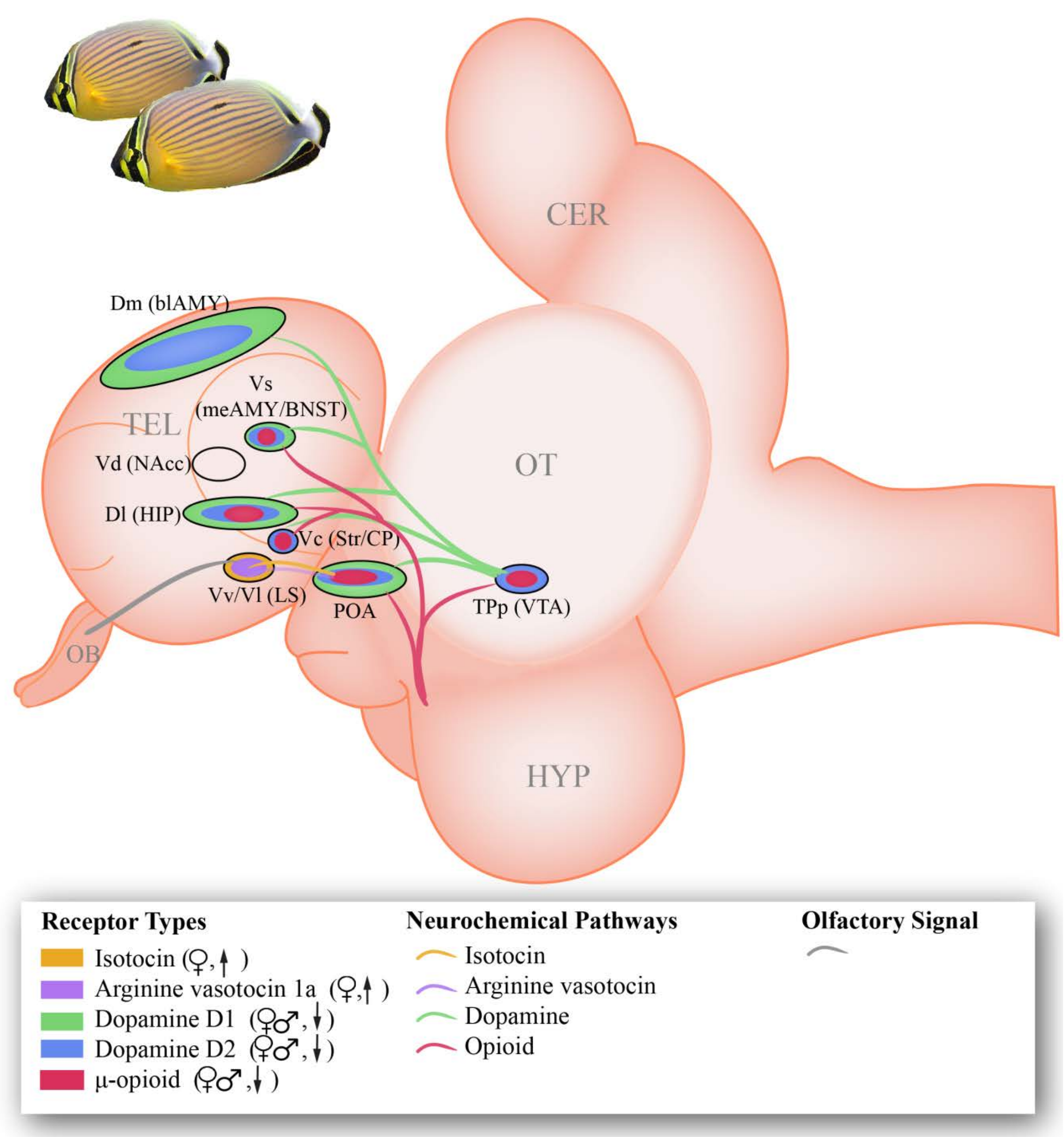


Figure 3.8. Sagittal view of brain illustrating a working neural network model for pair bonding in *Chaetodon lunulatus*. Colors within brain regions represent putative sites of action for each system based on comparative data on receptor gene expression provided here. Symbols and arrows indicate the sex(es) to which receptor phenotypes apply, and direction of receptor gene expression (up- or down-regulated) within brain regions, respectively. Colored lines represent putative neurochemical projections from predominant sites of synthesis (based on literature) to putative target sites (based on current findings). Illustration made by J.P.N., adapted from Dewan and Tricas, 2014, with permission.

3.5.5 Limitations and future directions

This is one of the first studies to explore the neurobiology of pair bonding in an early vertebrate (i.e., a reptile or an anamniote). While few other studies have researched the involvement of nonapeptides (i.e., teleosts: AVT: Dewan et al., 2008, 2011; Oldfield and Hofmann, 2011; O'Connor et al., 2016; IT: Oldfield and Hofmann, 2011; O' Connell et al., 2012), that I am aware of, this is the first to research the involvement of the dopamine and opioid systems, and examine gene expression in specific brain regions. I provide functional support for the involvement of these neurochemicals and their conjugate receptors (D1R and MOR notwithstanding). However, given that nonapeptide receptor antagonists were delivered peripherally, and that nonapeptide receptors exert multiple functions both centrally and peripherally (Lee et al., 2009; Goodson and Thompson, 2010), it is possible that treatment(s) did not pass the blood-brain barrier to act on the brain, and instead acted peripherally. Alternatively, given that peripheral and central actions of nonapeptides appear to be tightly integrated (Goodson and Thompson, 2010), it is possible that treatment initially acted upon peripheral receptors, resulting in downstream effects on central receptors in order to ultimately mediate behavior (Reddon et al., 2014). Additionally, my data on the brain regions in which neurochemical systems might act are only correlative and limited in scope. Furthermore, due to the paucity in neural research on fish pair bonding, I have relied heavily on drawing upon the rich body of literature on the mammalian model, *Microtus ochrogaster,* in order to speculate the cognitive and behavioral functions that these putative neural circuits might subserve. Finally, the sample sizes used in the current study are relatively small. Therefore, it is important to consider that my proposed neural network model of teleost pair bonding is far from conclusive and is certainly incomplete. Nonetheless, I believe it provides a useful foundation from which specific hypotheses related to teleost pair bonding can be tested in the future. A priority should now be to experimentally validate whether the neuroanatomical correlates of pair bonding found here are functionally relevant through undertaking brain-region specific manipulations (e.g., pharmacological), and if so, then whether these functions are analogous to those of mammalian counterparts. Furthermore, I advocate exploring the potential involvement of other promising brain regions that are critical to vertebrate social behavior, including the periaqueductal gray/central gray (PAG/CG), ventral tuberal nucleus (vTn) (homologous to the mammalian anterior hypothalamus, (AH)) and anterior tuberal nucleus (aTn) (homologous to the mammalian ventromedial hypothalamus, (VMH)) (Newman, 1999; Goodson, 2005; O'Connell and Hofmann, 2011). Moreover, similar preliminary investigations into the involvement of other likely candidate systems that modulate reward and positive reinforcement behavior (e.g., serotonin (Young et al., 2011) and orexin (Tsujino and Sakurai, 2013)), negative reinforcement behavior (i.e., corticotrophin releasing factor (CRF) (Lim et al., 2007; Bosch et al., 2009)), and motor output (e.g., GABAergic and glutamatergic) (Young and

Wang, 2004; Grillner et al., 2013)) are encouraged. Finally, in order to better understand the extent to which neurobiological systems of pair bonding have converged across vertebrate evolution, complementary research needs to be done on multiple species within and across all major taxonomic groups (Goodson, 2013).

3.5.6 Conclusions

In addition to representing an integral part of the human experience (Young and Wang, 2004; Young et al., 2005; Quinlan and Quinlan, 2007; Quinlan, 2008; Fletcher et al., 2015), pair bonding has independently evolved in every major vertebrate lineage. It is already clear that this has not occurred through a compete and universal convergence of a single regulatory neural network. However, my study contributes to an emerging pattern that in at least selective cases, even those involving phylogenetically distant taxa with distinct evolutionary histories, this might occur through at least a partially converged neural network. *M. ochrogaster* rodents and *C. lunulatus* teleosts, despite being separated by ~450 million years of independent evolution (Kumar and Hedges, 1998) and despite having opposing parental evolutionary histories (parental vs. non-parental, respectively), appear to share some striking similarities in the neural substrates that underpin pair bonding. I have discovered evidence for the involvement of isotocin, arginine vasotocin, dopamine, and opioid systems in *C. lunulatus* pair bonding, corresponding to their (or their homologs) involvement in *M. ochrogaster* counterparts. Moreover, I have described that in association with pair bonding sociality, nonapeptide receptor expression varies in the lateral septum-like region, while dopamine and opioid receptor expression varies within other regions of the mesolimbic reward network, including the striatum; mirroring sites of action in *M. ochrogaster*. Therefore, I tentatively suggest that the neurobiology of pair bonding between these taxa has at least partially converged through the repeated co-option of evolutionarily deep molecular and anatomical homologies that were already established in ancestral osteichthyes ~ 450 MYA. In order to determine the extent to which this has occurred across vertebrates, complementary studies across a wider range of lineages (most urgently amphibians and reptiles) are now needed.

Chapter 4: Endurance of pair bonding reduces intra-pair conflict and promotes cooperative territory defense among coral feeding butterflyfishes

4.1 Abstract

Pair bonding is generally linked to monogamous mating systems, where the reproductive benefits of extended mate guarding or of bi-parental care are suggested to be key adaptive functions. However, in coral-feeding butterflyfishes (f: Chaetodontidae, g: *Chaetodon*), pair bonding occurs between sexually immature and homosexual partners, and in the absence of any parental care. This suggests that there must be alternative adaptive benefits of pair bonding. In this study, I tested the hypothesis that coral-feeding pair bonding butterflyfishes cooperate in defense of food, conferring direct benefits for one or both partners. I provide evidence that partners of *Chaetodon lunulatus* and *C. baronessa* use alternative cooperative strategies during feeding territory defence. In *C. lunulatus,* both partners mutually defend their territory, while in *C. baronessa,* males prioritize territory defence; conferring improvements in feeding and energy reserves in both sexes relative to solitary counterparts. I further show that partner fidelity contributes to this function by showing that re-pairing invokes intra-pair conflict and inhibits cooperatively-derived feeding benefits, and that partner endurance is required for these costs to abate. Taken together, my results support the hypothesis that in coral-feeding butterflyfishes, pair formation and endurance enhances cooperative defense of prey resources, ultimately benefiting both partners by enabling greater resource acquisition and improving physiological condition.

4.2 Introduction

Pair bonding has independently evolved numerous times, and in all major vertebrate lineages (Reichard and Boesch, 2003), including mammals (9 % of species, Lukas and Clutton-Brock, 2013), birds (90 % of species, Lack, 1968; Cockburn, 2006), reptiles (Bull, 2000), amphibians (Gillette et al., 2000; Brown et al., 2010), fishes (Whiteman and Côté, 2004; Brandl and Bellwood, 2014), and invertebrates (Singer and Reichert, 1995; Mathews, 2002). Available data suggests that with few exceptions (birds: see Griffith et al., 2002; reptiles: Uller and Olsson, 2008; reef fishes: Brandl and Bellwood, 2014) pair bonded organisms are also reproductively monogamous (mammals: Clutton-Brock and Isvaran, 2006; birds: Griffith et al., 2002; reptiles: Uller and Olsson, 2008; amphibians: Brown et al., 2010; marine fishes: Whiteman and Côté, 2004; Brandl and Bellwood, 2014), which is defined as displaying disproportionately frequent (Barlow, 1984, 1986) if not exclusive (Wittenberger and Tilson, 1980) mating between a single male and a single female.

Two main hypotheses have been put forward to explain the evolution of pair bonding. The first hypothesis suggests that it results from direct selection for monogamous mating (Emlen and Oring, 1977; Wittenberger and Tilson, 1980; Barlow, 1984; Reavis and Barlow, 1998; Whiteman and Côté, 2004; but see Gwinner et al., 1994; Black et al., 2001; Mathews, 2002; Pratchett et al., 2006; Brandl and Bellwood, 2013). Specifically, monogamy may be favored due to the reproductive benefits of extended male mate-guarding (Seibt and Wickler, 1979; Brotherton and Manser, 1997; Reavis and Barlow, 1998; Lukas and Clutton-Brock, 2013). If females are too widely dispersed (Emlen and Oring, 1977; Kleiman 1977; Clutton-Brock and Harvey, 1978; Wickler and Seibt, 1981; Herold and Clark, 1993; Palombit, 2000; Lukas and Clutton-Brock, 2013), are intolerant of each other (Wittenberger and Tilson, 1980), or are rarely sexually receptive (Mathews, 2003), this might select for males to guard available females throughout a period of time that extends beyond reproductive events/female sexual receptivity, thus effectively mating monogamously. Secondly, it has been hypothesized that pair bonding results from selection for bi-parental care (Kleiman, 1977; Wittenberger and Tilson, 1980; Brown et al., 2010, McGraw et al., 2010), but empirical support has been largely limited to birds (Møller, 2003) and some mammals (e.g., Gubernick and Teferi, 2000). Under certain environmental constraints, both pair members may confer higher reproductive output by investing in mutual offspring (i.e., by provisioning resources to young (Lack, 1968; Wittenberger and Tilson, 1980; Brown et al., 2010), or protecting them from infanticide (Opie et al., 2013)) than by seeking extra-pair reproductive opportunities (Kleiman, 1977; Wittenberger and Tilson, 1980).

Aside from monogamous mating and bi-parental care, pair bonding might be attributed to the benefits of social assistance during ecological (including non-reproductive) processes that are directly conferred to one or both partners (Black, 1996; Pratchett, 2006). One such process that may benefit from pair bonding is cooperative defense of high value resources, such as nesting sites, food, or shelter holes (Rutberg, 1983; Wilson, 2000). Two modes of defense assistance are typically recognized. Most commonly, resources are defended primarily or exclusively by males [referred to as male-prioritized "division of labor" (Eduard and Linsenmair, 1971; Hourigan, 1987, 1989; Vaughan and Vaughan, 1986), or "resource brokering" (Gowaty, 1996; Wrangham, 1976)] in order to alleviate females from this duty. The benefits of such division of labour are presumably related to increased mating access to females (Tecot et al., 2016) or fitness benefits arising from increased fecundity of females (Hourigan, 1987, 1989; Morley and Balshine, 2002; Black et al., 2014), or for other resources/services that are partitioned by females (Eduard and Linsenmair, 1971; Linsenmair, 1984; Mathews, 2002). Less commonly, resources are mutually defended, or "co-defended" by male and female partners (Fricke, 1986; Tecot et al., 2016), presumably because both sexes directly benefit by sharing this responsibility (Tecot et al., 2016).

The assisted or cooperative resource defense hypothesis (ARDH) makes several fundamental predictions about pair formation and bonding: 1) pairs persist outside of breeding periods (Tecot et al., 2016); 2) pairing frequency increases with resource value (Tecot et al., 2016); 3) males primarily defend resources within a territory (Rutberg, 1983; Whiteman and Côté, 2004; Tecot et al., 2016), where (a) they respond to all intruders, and females only respond toward female intruders, and (b) females are unable to maintain a territory alone or directly benefit from males assistance (Whiteman and Côté, 2004); or 4) partners mutually defend resources within a territory (Rutberg, 1983; Mathews, 2002; Whiteman and Côté, 2004; Tecot et al., 2016) where (a) both sexes respond agonistically towards intruders of both sexes, and (b) individuals are unable to maintain a territory alone or directly benefit from each other's assistance (Mathews, 2002; Whiteman and Côté, 2004). Although the role of ARD in promoting pair bonding has received considerably less research attention than extended mate-guarding or bi-parental care, *in situ* observations and empirical tests of these predictions have provided support for this hypothesis across a wide range of taxa (**Table 4.1**) (but see: Gibbon, 1997; Hilgartner et al., 2012).

With few exceptions (i.e., in pink flamingos, who exhibit transient partnerships: King, 2006), species that appear to pair at least in part for assisted resource defense display long-term partner fidelity, persisting with their partner from many months and sometimes throughout their life-time (**Supplementary Table 4.1**). This is especially apparent among species who display a high degree of site fidelity (Ens et al., 1996). Several hypotheses might explain pair bond endurance, as opposed to transient pairing, within the context of assisted resource defense. Pairs might endure because this improves resource defense assistance (Black, 2001; Black et al., 2014) and/or reduces intra-pair conflict (Eduard and Linsenmair, 1971; Linsenmair, 1984). Partners might also endure if there is a delay in the time at which they reciprocate resource/service provisioning towards each other (Whiteman and Côté, 2004). For example, if male assistance is based on increasing female feeding investment in order to share her improved fecundity, then males may remain with females across reproductive periods if there is a time-lag between enhanced female feeding and egg production (Whiteman and Côté, 2004). Finally, pair endurance might emerge from mutual site-attachment to the valued resource, which may arise if the valued resources is scarce or costly to acquire. These hypotheses are not mutually exclusive.

Corallivorous (coral-feeding) butterflyfishes of the genus *Chaetodon* are ideal subjects for testing the ARDH for pair bonding, as well as testing the underlying basis of pair bond endurance. This speciose group of teleost fishes inhabits tropical coral reefs (Burgess, 1978; Allen, 1979), where numerous species with diverse sociality exist in sympatry (Chapter 1). Moreover, pairing occurs despite the absence of parental care

(Neudecker and Lobel, 1982; Fricke, 1973; Driscoll and Driscoll, 1988). *In situ* mating observations indicate that heterosexual pairs of at least some species (mainly, *Chaetodon lunulatus*) are reproductively monogamous (Neudecker and Lobel, 1982; Fricke, 1986, Hourigan, 1989, Yabuta, 1997) and display mate-guarding behavior (Fricke, 1986; Hourigan, 1989). However, same-sexed (*C. multicinctus:* Tricas, 1986; *C. lunulatus:* Pratchett et al., 2006; Nowicki, unpublished data, *C. capistratus*: Gore, 1983, *C. melannotus*: Pratchett et al., 2006; *C. baronessa*: Nowicki, unpublished data) reproductively immature (Tricas and Hiromoto, 1989; Fricke, 1986, Pratchett et al., 2006) and reproductively inactive (Fricke, 1986, Yabuta, 2007) pairing also occurs in these organisms. Moreover, available data suggests that chaetodontids exhibit a protracted mating season (Tricas, 1986; Lobel, 1989; Yabuta, 1997), so it is unlikely that females are rarely sexually receptive. As such, pairing is unlikely to have a reproductive basis, or at least did not arise as a direct consequence of bi-parental care or extended mate-guarding.

Butterflyfishes display a variety of social systems that are often correlated with different feeding guilds (Reese, 1975, Fricke, 1986). Twenty-four *Chaetodon* species are reported to feed predominantly (≥ 80%), if not exclusively on scleractinian corals (Pratchett, 2014). Coral has a poor energetic value (Tricas, 1989b), and is inefficiently assimilated by butterflyfishes (Hourigan, 1987, 1989), yet it is temporally and spatially stable, and therefore economically defendable (Tricas, 1985, 1989; Hourigan, 1989). Corallivorous species are more likely to be territorial than planktivorous or generalist omnivores (Roberts and Ormond, 1992)—species whose diets may have a relatively higher nutrient value, and in the case of plankton is not defendable (Hourigan, 1987, 1989). Likewise, interspecific dominance over feeding sites increases with dietary specialization, such that obligate corallivores dominate territorial disputes over feeding generalists (Blowes et al, 2013). In association, corallivorous species tend to be pair bonding, whereas planktivorous species are mostly gregarious, while feeding generalists exhibit varying social systems (reviewed by Roberts and Ormond, 1992). Given these closely-linked ecological attributes, for corallivorous species, dietary energy assimilation per bite is suggested to limit fitness, especially for females, who invest considerable energy into gamete production (Tricas, 1989; Hourigan, 1989); and pairing is suggested to arise from the need for assisted defense of coral prey against conspecific and heterospecifics in order to better invest in feeding and energy reserves (Fricke, 1986; Hourigan, 1987; Roberts and Ormond, 1992). However, in these organisms, predictions for the ARDH have rarely been tested (but see: Fricke, 1986; Hourigan, 1987), and evidence that assistance confers energy reserve gains remains entirely absent. Furthermore, pair bonding butterflyfishes are presumed to have very high levels of partner fidelity based on repeated observations of pairing between specific tagged or individually recognizable individuals over periods of up to seven years (**Supplementary**

Table 4.1). However, the ecological basis of pair bond fidelity among these organisms remains unresolved.

The overall aim of this chapter was to test whether pair bonding in two species of coral-feeding butterflyfishes (*C. lunulatus* and *C. baronessa*) may be attributed to benefits of assisted resource defense directly conferred to one or both partners. Moreover, I wanted to explicitly test whether endurance of pair bonding (and therefore mate familiarity) enhances the effectiveness of co-operative resource defense. Specifically, I aimed to test ARDH predictions that either: 1) males primarily defend the feeding territory (a), and females benefit from male assistance by improved investment in feeding and energy reserves (b), or 2) both partners mutually defend their feeding territory (a) and benefit from each other's assistance by improved investment in feeding and energy reserves (b). If so, then finally, I tested the prediction that 3) pair bond promotes pair-wise assistance during territory defense and/or reducing intra-pair conflict. To test ARDH predictions 1a and 2a, I conducted *in situ* observations on naturally occurring pairs, characterizing their level of intra-pair coordination, and comparing levels of agonism towards competitors between male and female partners. To test predictions 1b and 2b, I conducted additional *in situ* observations on naturally occurring pairs and solitary individuals, examining whether paired individuals had higher per capita coral cover within their territory, displayed lower agonism towards competitors, and higher feeding strikes and liver hepatocyte vacuole density than solitary counterparts. To test prediction 3, I determined whether assisted territory defence (represented by intra-pair coordination) and intra-pair agonism varied with pair endurance. In association, I examined whether per capita agonism towards conspecifics and congenerics declines, while feeding strikes and physiological condition increases, with pair endurance. This was achieved by first monitoring these attributes among naturally occurring, enduring pairs in order to establish "base-line" levels. Thereafter, I experimentally induced new partnerships by removing one of the original partners, and re-monitored these attributes as new partnerships persisted through time. To measure effectiveness of territory defence and benefits accrued from cooperative resource defence, I recorded the number of agonistic acts by focal individuals towards conspecifics and heterospecifics (along with intra-pair agonism) assuming that such agonism entails a cost to the individual, such that time and energy is divested from feeding. The benefits of effective resource defence are thus inferred based on individual bite rates, whereby higher bite rates would reflect both increased time available for feeding and lower levels of individual vigilance.

To estimate the fitness benefits accrued from such changes in agonistic and feeding behaviour, I measured densities of liver hepatocyte vacuoles. In butterflyfishes, liver hepatocyte vacuole density is directly proportional to liver lipid content (Pratchett et al., 2004). In fishes, lipid is the favoured energy reserve, and the liver is generally the first site of lipid storage (Cowey and Sargent, 1977). Because liver lipid stores are rapidly

mobilized during high energy expenditure (Black and Love, 1986), or reduced food intake (Green and McCormick, 1999; Pratchett et al., 2004), they (and thereby hepatocyte vacuolation) represent a sensitive proxy for individual body condition and subsequent fitness (Pratchett et al., 2004).

Table 4.1. Taxa hypothesized to pair bond for assisted resource defence (ARD) purposes.

Taxon	Evidence for assisted resource defence (ARD)* (Resource type)	Mode of ARD	Reason(s) for ARD	Partner fidelity	Reason(s) for partner fidelity
Mammals					
Eulemur rubriventer	(Food) [1]: 1. Pairs stable and persist without reproductive activity [1] 2. Pair bond frequency and/or pair territoriality varies with resource availability [1] 3. Pairs work together or separately to defend resources [1]	Mutual [1]	Unknown	≥ 6 years [1]	Unknown
Lavia frons	(Food) [2]: 1. Pairs stable and persist without reproductive activity [2] 2. Pairs work together or separately to defend resources [2]	Male-exclusive [2]	Improve energy budget of pair (untested) [2]	≥ 1 year [2]	Unknown
Castor fiber	(Food) [3]: 1. Pairs stable and persist without reproductive activity [3] 2. Pairs work together or separately to defend resources [3]	Male-prioritized [3]	Secure food for females and offspring (untested) [3]	Long-term [3]	Unknown
Birds					
Anser anser	(Food) [4, 5]: 1. Pairs stable and persist without reproductive activity [4, 5] 2. Pairs work together or separately to defend resources [4, 5]	Mutual [4]	Improve competition, feeding, and survival (tested, supported) [4]	Long-term [6]	Unknown
Peucaea ruficauda	(Food, water) [7]: 1. Pairs stable and persist without reproductive activity [7]	Female-prioritized [7]	Unknown	≥ 1 year [7]	Unknown
Branta leucopsis	(Food, nesting sites) [8]: 1. Pairs stable and persist without reproductive activity [8] 2. Pairs work together or separately to defend resources [8]	Male-prioritized [8]	Improve feeding, energy reserve, and reproduction in females (feeding tested, supported) [8]	1 year-life-long [8]	Improves cooperative food acquisition and reproduction of pair (tested, supported) [8]

Fish					
Eretmodus cyanostictus	(Food, shelter) [9]: 1. Pairs stable and persist without reproductive activity [9] 2. Pairs work together or separately to defend resources [9]	Male-prioritized [10]	Increase territory acquisition in females (tested, supported) [9]	Long-term [10]	Unknown
Chaetodon chrysurus (= *paucifasciatus*)	(Food) [11]: 1. Pairs stable and persist without reproductive activity [11] 2. Pairs work together or separately to defend resources [11]	Mutual [11]	Reduce territory defence and improve feeding (tested, supported) [11]	Months-years [11]	Unknown
Chaetodon multicinctus	(Food) [12, 13]: 1. Pairs stable and persist without reproductive activity [12, 13] 2. Pairs work together or separately to defend resources [12, 13]	Male-prioritized [12, 13]	Reduce territory defence in both sexes and improve feeding in female (tested, supported) [12, 13]	Months-years [12]	Unknown
Chaetodon quadrimaculatus	(Food) [12, 13]: 1. Pairs stable and persist without reproductive activity [12, 13] 2. Pairs work together or separately to defend resources [12, 13]	Male-prioritized [12, 13]	Reduce territory defence in both sexes and improve feeding in female (tested, supported) [12,13]	1 year–long-term [14]	Unknown
Chaetodon lunulatus	(Food) [**] 1. Pairs stable and persist without reproductive activity [**] 2. Pairs work together or separately to defend resources [**]	Mutual [**]	Improve feeding and energy reserves in both partners (tested, supported) [**]	≥ 7 years [15]	Improves cooperative food defence and reduces conflict between partners (tested, supported) [**]
Chaetodon baronessa	(Food) [**] 1. Pairs stable and persist without reproductive activity [**] 2. Pairs work together or separately to defend resources [**]	Male-prioritized [**]	Improve feeding and energy reserves in both partners (tested, supported) [**]	≥ 4 months [16]	Improves cooperative food defence and reduces conflict between partners (tested, supported) [**]

Invertebrates					
Hemilepistus reaumuri	(Burrow) [17, 18]: 1. Pairs stable and persist without reproductive activity [17, 18] 2. Pairs work together or separately to defend resources [17, 18]	Male-prioritized,[17]	Females forage without losing burrow (untested)[17]	Unknown	Unknown
Alpheus angulatus	(Burrow) [19]: 1. Pairs work together or separately to defend resources [19]	Male-prioritized [19]	Reduces risk of female eviction (tested, supported) [19]	Unknown	Unknown

References: [1] Tecot et al., 2016; [2] Vaughan and Vaughan, 1986; [3] Rosell and Thomsen, 2006; [4] Kotrschal et al., 2006; [5] Kirschenhauser, 2012; [6] Kotrschal et al., 2010; [7] Illes, 2015; [8] Black et al., 2014; [9] Morley and Balshine, 2002; [10] Morley and Balshine, 2003; [11] Fricke, 1986; [12] Hourigan, 1987; [13] Hourigan, 1989; [14] Driscoll and Driscoll, 1988; [15] Reese, 1991; [16] Reese, 1973; [17] Eduard and Linsenmair, 1971; [18] Linsenmair, 1984; [19] Mathews, 2002

Notes: *Predictions of ARDH for pairing put forth by Whiteman and Côté, 2004; Tecot et al., 2016 ** Findings from current study

4.3 Methods

4.3.1 Study location and model species

This study was undertaken on adjacent sheltered reefs of Lizard Island, located northern section of the Great Barrier Reef, Australia (14°40'S, 145°27'E) from January 6 - March 23, 2014. Sampling was undertaken using the two most abundant coral-feeding and pair bonding butterflyfishes, *C. lunulatus* and *C. baronessa* (Chapter 2) (**Figure 4.1**). Both species are territorial (Reese, 1981; Yabuta, 1997, 2000; Berumen and Pratchett, 2006; Blowes et al., 2013) and are predominantly found in long-term, heterosexual pairs (Reese, 1973; Yabuta, 1997; Chapter 2). Only adult sized individuals were examined in the current study (*C. lunulatus*: > 64 mm standard length (SL); *C. baronessa*: > 61mm SL).

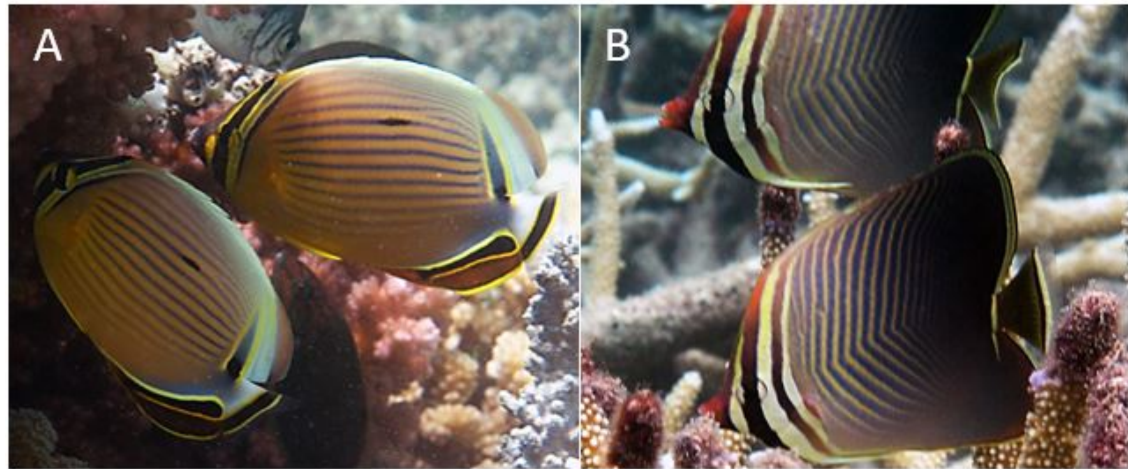


Figure 4.1. Model species of pair bonding butterflyfishes used in the current study. At the study location, Lizard Island (GBR), *C. lunulatus* (A) and *C. baronessa* (B) are highly territorial coral feeding specialists that display enduring pair bonds. Pictures are of focal pairs used in this study, taken by J. P. N.

4.3.2 Coordination and competitor agonism between male and female partners

To test whether pairs displayed either male-prioritized or joint territory defence, I conducted *in situ* observations on naturally occurring pairs, characterizing their level of intra-pair coordination, and comparing levels of agonism towards competitors between male and female partners. Pair bonded individuals were haphazardly encountered and identified as two individuals that displayed coordinated swimming exclusively with each other during a five-minute observation. Care was taken to sample diffent reef sites in order to avoid re-sampling the same pairs. Coordination, defined as the synchronisation of individuals' movements in space and time (Herbert-Read, 2016), was quantitatively identified here as the focal fish being positioned within a 2-m distance from its partner whilst being faced within a 315-45° angle relative the faced position of its partner (designated as 0°) (**Figure 4.2**). After confirming paired sociality, individuals were

observed from a distance of 2-4 metres and allowed three minutes to acclimate to observer presence. Thereafter, *in situ* observations were conducted for 6-minutes, to record pair coordination and territorial defense. All studies were conducted on snorkel between 08:30-17:30hr. Levels of pair coordination were determined by recording the presence or absence of pair coordination every ten-seconds. For each individual, rates of agonistic behavior were quantified as the total number of agonistic acts towards conspecifics and congenerics. Agonistic acts that were observed and quantified included staring, head down, tail-up display, chasing, fleeing, and encircling (see Yabuta, 2000 for detailed ethogram). Agonism was only recorded if it was directed towards other butterflyfishes, based on the observation that most butterflyfish territorial competition is intra-familial (Wrathall et al., 1992). After each observation, both individuals were collected by spearing through the dorsal musculature and sacrificed in an ice slurry for sex determination.

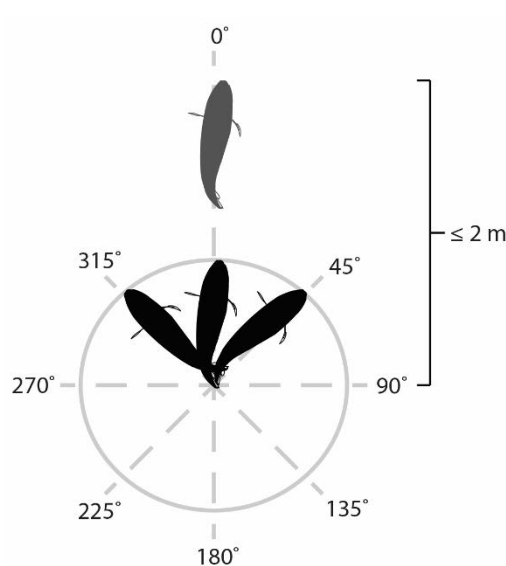


Figure 4.2. Coordinated swimming examined in paired butterflyfish. Coordinated swimming by focal fish (black) was defined as being positioned within a 2-m distance from its partner (grey) whilts being faced within a 315-45° angle relative the the faced position of its partner (designated as 0°).

4.3.3 Paired vs. solitary individuals

4.3.3.1 Per capita competitor agonism and feeding strikes

To test whether one or both sexes benefit from pairing by reduced competitor agonism, increased feeding rates or increased access to preferred coral prey, I compared these variables between naturally occurring paired and solitary individuals of both sexes. Individuals were considered pair bonded using the criteria previously described, and they were considered solitary if they displayed no coordinated swimming with another individual during a five-minute observation period. After establishing their social status and undergoing 3-minute acclimation to observer presence, focal individuals underwent a single 6-mintue observation to record: i) total feeding bite rate, determined by the number of bites taken on any coral ii) total feeding bites on preferred coral types (data only collected for *C. baronessa*, whose preferred coral food is *Acropora hyacinthus, A. florida,* and *Pocillopora damicornis* (Berumen et al., 2005; Lawton et al., 2012)), and iii) rates of agonism. Given that rates of agonism may be affected by the local densities of competitors (independent of levels of agonism exhibited by focal individuals), the number of agonistic acts recorded during replicate observations was standardized to

account for the densities of potential competitors (conspecific and congenerics) located within the immediate vicinity of their feeding territory.

4.3.3.2 Per capita procured coral food supply

Variation in *per capita* food availability within territories of paired versus solitary conspecifics was estimated for both species. The territory boundary for each pair or individual was estimated by marking the exact position of the focal individual(s) at regular individual through the course of the observation. The outermost limits of where each individual or pair were located were then considered to roughly correspond with their territorial limits. To estimate percent coral cover within territories, I ran 2-4 replicate 2-metre point-intercept transects within the boundary, run from haphazardly selected starting points. In cases where estimated territory size was small, it was only possible to run a maximum of 2 replicate transects. For each replicate transect, I recorded the substrate (focussing on availability of different coral species) underlying each of 20 points per transect. Since food availability is theoretically shared equally between paired individuals, per capita availability of overall coral prey and preferred coral prey was determined by dividing total cover of these categories by two prior to analysis. After recording the behavior of focal individuals and measuring food availability, individual butterflyfishes were collected by spearing through the dorsal musculature and sacrificed in an ice slurry for sex determination and body condition analysis.

4.3.4 Enduring vs. new pairs: Intra-pair relations, and per capita competitor agonism and feeding strikes

I used a partner removal-replacement experiment to examine whether pair bond endurance reduces territory defense or increases feeding of paired individuals by promoting cooperative territory defense or reducing intra-pair conflict. Naturally occurring pair bonds of *C. lunulatus* (n = 9) and *C. baronessa* (n = 10) were identified among individuals on 3 adjacent sheltered reefs using methods previously described. Pairs were assumed to have been enduring, based on previous research showing a high level of partner endurance in these species at the study location (Chapter 2). Prior to experimentation, one individual from each pair was haphazardly chosen as the focal individual for the experiment. To identify the focal individual and its partners throughout the experiment, a high definition photograph of both sagittal sides of their body was taken, from which a unique set of body markings were recognized and used for repeated individual identification (Yabuta, 1997). Behavioral expression of the focal individual while with its original partner was measured throughout an eight-minute observation, for five consecutive days. Prior to each observation, the focal individual and its partner acclimated to observer presence (as described above). During each

observation, time spent coordinatedly swimming with partner, agonism towards partner, agonism per competitor, and feeding bites of the focal individual were recorded using the methods previously described. Immediately following observations conducted over five consecutive days, the partner of the focal individual was removed via spearing and sacrificed in an ice slurry for sex determination and body condition analysis. Within 24 hours of experimental partner removal, all focal individuals had naturally re-paired with a new partner with whom they remain paired for the remainder of the study (with the exception of one individual, see result section 4.4.3). I then conducted the same behavioral observations for a further seven consecutive days (in the case of *C. lunulatus*) or eight days (in the case of *C. baronessa*). After experimentation, the focal individual and its new partner were collected by spearing through the dorsal musculature and sacrificed in an ice slurry in order to determine the sex of both individuals and body condition of the focal individual's new partner.

4.3.5 Sex determination

The sex of focal fish was determined histologically. Gonads were removed and fixed in formaldehyde-acetic acid-calcium chloride (FACC) for at least 1 week. thereafter, gonads were dehydrated in a graded alcohol series, cleared in xylene, embedded in paraplast, sectioned transversely (7 µM thick), and stained with hematoxylin and eosin. Sections were examined under a compound microscope (400 X magnification) for the presence of sperm (male) or oocytes (female) (Pratchett et al., 2006).

4.3.6 Solitary vs. newly paired vs. enduringly paired individuals: Differences in liver hepatocyte vacuolation

To assess changes in energy reserves in association with pairing and partner endurance, I compared liver hepatocyte vacuole density between individuals who naturally occurred in solitude (liver specimens acquired from *in situ* observation study), who were in new pair bonds (*C. lunulatus*: five day old partnerships; *C. baronessa*: seven day old partnerships), and who were in naturally occurring enduring pair bonds (liver specimens for the latter two conditions were acquired from individuals from partner removal experiment). Whole livers were dissected and fixed in 4% phosphate-buffered formalin (PBF). Fixed liver tissues were then dehydrated in a graded ethanol series and embedded in paraffin wax blocks. Tissues were sectioned at 5µm, mounted onto glass slides, and stained using Mayer's hematoxylin and eosin to emphasize hepatocyte vacuoles. Hepatocyte vacuole density was then quantified using a Weibel eyepiece to record the proportion of points (out of 121) that intersected with hepatocyte vacuoles when viewed at X 40 magnification. Three estimates of hepatocyte vacuolation were taken for each of 3 haphazardly chosen cross sections, totaling 9 replicate estimates per

fish liver; from which the mean proportion of vacuoles in liver hepatic tissues of each fish was calculated, following Pratchett et al. (2004).

4.3.7 Statistical analysis

4.3.7.1 Coordination and competitor agonism between male and female partners

A one-way ANOVA was used to compare the level of coordinated pair swimming between *C. lunulatus* and *C. baronessa*. Coordinated swimming data was square-root transformed prior to analysis to improve normality of residual variance. For both species, a one-way ANOVA was used to compare rates of agonism between sexes.

4.3.7.2 Paired vs. solitary individuals: Differences in competitor agonism, bite rates, and access of coral prey

For each sex of each species, rates of agonism were compared between social conditions using non-parametric Mann-Whitney *U* tests, due to non-normal distribution of residual variance. For each species, feeding bite rate on total coral was compared between social conditions using a factorial ANOVA (with sex and social condition as fixed factors). For each sex of *C. baronessa,* a non-parametric Mann-Whitney *U* test was used to compare feeding bite rate on preferred coral between social conditions, due to non-normal distribution of residual variance. For each sex of *C. lunulatus,* per capita percentage of total coral cover within territory was compared between social conditions using a non-parametric Mann-Whitney *U* test. For *C. baronessa,* a factorial ANOVA (with sex and social condition as fixed factors) was used to compare per capita percentage of total coral cover within territory between factor levels, and a non-parametric Mann-Whitney *U* test was used to analyze this data separately for each sex.

4.3.7.3 Enduring vs. new pairs: Intra-pair relations, and per capita competitor agonism and feeding strikes

Temporal changes in time spent coordinatedly swimming with partner, agonism towards partner, agonism per competitor, and feeding bites were analyzed using multivariate analysis of variance (MANOVA), with results displayed using canonical discriminant analysis (CDA). In this context, MANOVA was used to examine whether the "union" of response variables, rather than the individual response of each variable, is significantly different between groups (Cruz-Castillo et al., 1994). CDA was then used to identify interactions within and among response variables and with treatments (Cruz-Castillo et al., 1994, 1997). More specifically, CDA reflects group differences to the greatest degree possible and generates canonical coefficients that yield relative information on each response variable in distinguishing between groups. This approach is preferable over using biometrical methods to evaluate the effects of relationship

"phase" (i.e., enduringly paired vs. newly paired) and "development time" (i.e., days together since becoming newly paired) on behaviors, as the latter approaches are limited to only focusing on "narrowly defined sectors of highly integrated systems involving inter-correlated variables" (Cruz-Castillo et al., 1997).

4.3.7.4 Solitary vs. newly paired vs. enduringly paired individuals: Differences in liver hepatocyte vacuolation

In both species, differences in the percentage of liver hepatocyte vacuolation between solitary, newly paired, and enduringly paired fish were analyzed using a non-parametric Kruskal-Wallis one-way ANOVA (Siegel and Castellan, 1988), due to non-normality in residual variance. Variation in hepatocyte vacuolation could not be analyzed for each sex separately, due to small sample sizes. Tukey and Kramer (Nemenyi) *post hoc* tests were used to identify differences between social condition means.

4.4 Results

4.4.1 Coordination and competitor agonism between male and female partners

The two-study species (*C. lunulatus* and *C. baronessa*) exhibited contrasting models of cooperative or shared territory defense. Pairs of *C. lunulatus* were highly conspicuous, where partners clearly spent the vast majority of their time swimming proximately (i.e., within a 2-meter distance) to each other throughout their feeding territory. While doing so, partners were continuously grazing on coral, and were often (i.e., ~ 56 % of the time) positioned in the same direction, coordinatedly swimming as one social unit. When encountering neighboring conspecifics and congenerics, agonism was minimal both in frequency and intensity, where it was mostly confined to “staring” or “head-down” displays. Importantly, both partners displayed equal levels of agonistic acts ($F_{1,12}$=1.01, p = 0.334; **Figure 4.3 a, b**), suggesting that there is mutual cooperation in territory defense. By contrast, *C. baronessa* partners spent notably less time swimming proximally to each other. When partners were within proximity, they also displayed considerably less coordination than *C. lunulatus*, ($F_{1,25}$=40.04, p = 0.000) spending only ~10% of their time coordinatedly swimming. In general, males tended to move over large distances within and along the boundaries of their territory whilst continuously foraging, whereas if territories contained a predominant outcrop of preferred coral, females tended to restrict movement to within that area whilst continuously foraging on the outcrop. If neighboring conspecific or congenerics were encountered, territorial disputes were infrequent and of low intensity. When territorial disputes did occur, however, *C. baronessa* males exerted 42% higher levels of agonism than females ($F_{1,8}$=7.51, p = 0.025; **Figure 4.3 a, c**), suggesting that in this species, territory defense is male-prioritized.

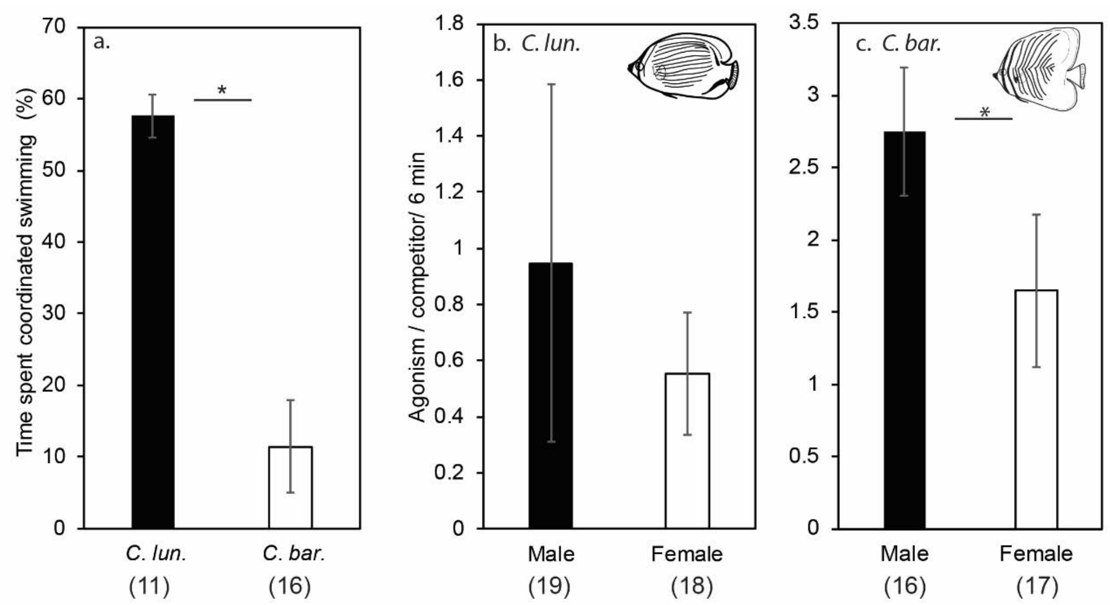


Figure 4.3. Patterns of pair coordination (a) and agonism towards competitors between male and female partners of *C. lunulatus* (b) and *C. baronessa* (c). Data are represented as the mean ± SEM; asterisks indicate statistically significant differences between treatment groups (ANOVA, $p < 0.05$). Sample sizes are listed below each treatment group.

4.4.2 Paired vs. solitary individuals: Differences in competitor agonism, food supply, and feeding strikes

4.4.2.1 Agonism per competitor rate

Across study sites, naturally occurring pairs of both species were common, whereas singletons were very rare. Pairs predominantly occurred at reef sites that were characterized by a high coral cover and (presumably as a consequence) high abundances of conspecifics and congenerics relative to solitary individuals. The higher abundance of neighboring conspecifics and congenerics surrounding paired individual's territories (in *C. lunulatus* by ~36%, in *C. baronessa* by ~75%) suggested that they had more neighboring competitors than solitary counterparts. After accounting for differences in competitor abundance, there was no apparent difference in rates of agonism between paired and solitary individuals for either species or gender (*C. lunulatus* males: z=-0.78, *p*=0.48; *C. lunulatus* females: z = -1.69, *p*=0.11; *C. baronessa* males: z=-.53, *p*=0.64; *C. baronessa* females: z=-0.00, *p*=1.0; **Figure 4.4 a, d**). Although paired individuals do exhibit mutual cooperation in territorial defense (albeit male-prioritized assistance in *C. baronessa*), there is no evidence that this reduces the time and investment involved in confronting or chasing potential competitors.

4.4.2.2 Food supply

For both species (*C. baronessa* and *C. lunulatus*), territories of paired individuals had higher coral cover than territories of solitary individuals. For *C. lunulatus,* territories of paired individuals had 50% higher coral cover compared to solitary individuals. Similarly, in *C. baronessa,* territories of paired individuals had 69% higher coral cover, and 99% higher cover of preferred coral prey (*A. hyacinthus, A. florida,* and *P. damicornis*, pooled) compared to territories of solitary individuals. However, if we assume that equal sharing of resources within pairs means that there is only half the coral prey available to each partner, it becomes apparent that for *C. lunulatus,* paired individuals have similar access to prey as solitary individuals (males per capita: z=-1.02, *p*=0.33; females per capita: z = -0.07, *p*=0.96; **Figure 4.4b**). By contrast, even after accounting for food sharing between *C. baronessa* partners, the territories of paired individuals still contained a higher proportion of total coral cover ($F_{1,41}$= 28.76*,* p = 0.00) and preferred coral cover (males per capita:, z=-2.32, *p*=0.03; females per capita: z=-2.06, *p*=0.04) per capita than those of solitary individuals. For females, territories held by single individuals contained 16.08 ± 1.20 SE percent total coral cover, and 0.75 ± 0.75 SE percent preferred coral cover; whereas paired females' territories contained 27.5 ± 1.30 SE percent total (approx. 43 % more), and 13.02 ± 3.7 SE percent preferred (approx. 94 % more) per capita. The same pattern was observed for *C. baronessa* males, where the territories of single males had 19 ± 2.15 SE percent total coral cover, and no preferred coral cover; whereas those of paired males had 27.66 ± 1.31 SE percent total coral cover (approx. 31 % more), and 13.02 ± 3.72 SE percent preferred cover (100 % more) per capita (**Figure 4.4e**). Overall, this suggests that pairing is associated with increased coral food supply procurement in both sexes of *C. baronessa,* whereas paired individuals of *C. lunulatus* do not benefit from great access to prey resources*.*

4.4.2.3 Feeding bite rate

In both sexes of both species, variation in bite rates suggest that paired individuals have higher rates of feeding and ingest more coral than solitary counterparts (in *C. lunulatus*: $F_{1,44}$= 28.57*,* p = 0.00; in *C. baronessa*: $F_{1,40}$= 28.91*,* p = 0.00, preferred coral strikes: z=-2.42, *p*=0.013 (males), z=-2.88, *p*=0.00 (females), **Figure 4.4 c, f**). In *C. lunulatus,* single males took 36.83 ± 8.87 SE bites per 6-min, whereas paired males took 86.21 ± 4.28 SE bites (approx. 57 % more); and single females took 37.4 ± 9.41 SE bites per 6-min, whereas paired females took 88.67 ± 8.56 SE bites (approx. 58 % more). Consistently, in *C. baronessa,* single females took 46 ± 3.21 SE total coral bites per 6 min, among which 1.17 ± 1.17 SE bites were on preferred coral; whereas paired females took 79.67 ± 4.91 SE total coral bites per 6 min, among which 33.33 ± 10.98 SE bites were on preferred coral (~43 % more total coral bites, and ~96% more preferred coral bites). Similarly, single males took 37.4 ± 3.93 SE total bites per 6 min, among which 1.4 ± 1.16 SE bites were on preferred coral; whereas paired males took 73.13 ± 4.86 SE total coral bites per

6-minutes, among which 32.44 ± 10.54 SE were on preferred coral (~49 % more total coral bites, and ~96 % more preferred coral bites).

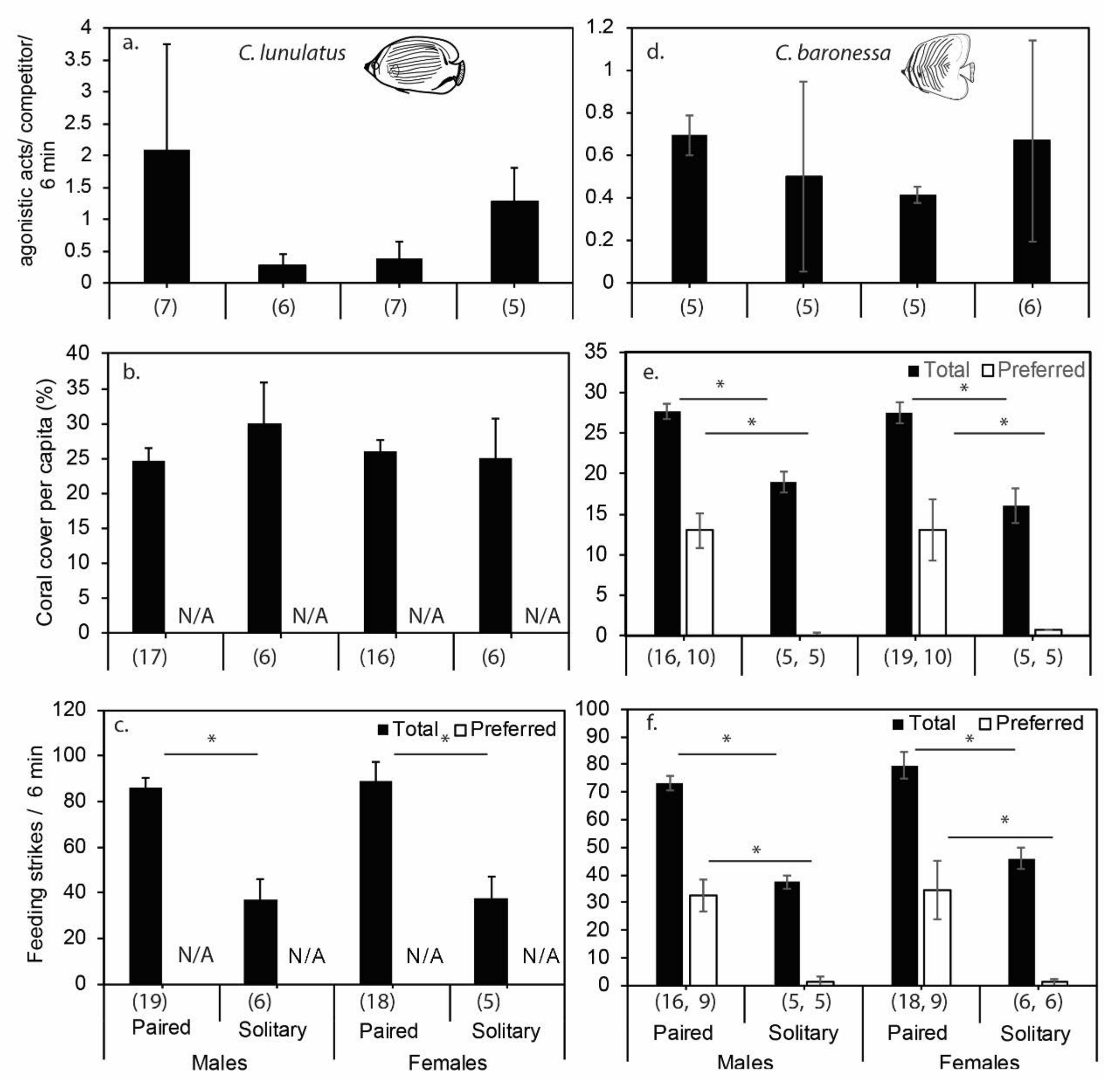


Figure 4.4. Differences in agonism towards competitors (a, d), coral food procurement (b, e), and bite rates (c, f) between paired and solitary *C. lunulatus* and *C. baronessa* individuals. Data are represented as the mean ± SEM; asterisks indicate statistically significant differences between treatment groups (ANOVA or Mann-Whitney *U*, $p < 0.05$). Sample sizes are listed below each treatment group.

4.4.3 Enduring vs. new pairs: Intra-pair relations, and per capita competitor agonism and feeding strikes

4.4.3.1 Costs of new pairing

Throughout the five consecutive days leading up to partner removal, all focal individuals maintained their same partner and territory. Within 18 hours of removing their original

partner, all focal individuals had kept their same territory, and had nonetheless re-paired. This indicates strong territory fidelity, and while there is strong pressure to be paired, this is not a result of partner/mate scarcity. After re-pairing, however, the activity profile (including union of pair swimming, within-pair agonism rate, competitor agonism rate, and feeding bite rate) of fishes dramatically changed (MANOVA: *C. lunulatus:* Pillai's trace = 0.23, df = 1, $p < 0.001$; *C. baronessa*: Pillai's trace = 0.29, df = 1, $p < 0.001$). In both species, the standardized canonical coefficients of CDF_1 were mostly influenced by high within-pair agonism, and to a lesser extent by coordinated swimming (in *C. lunulatus*: *low* coordinated swimming; in *C. baronessa*: *high* coordinated swimming), higher agonism per competitor, and low feeding bite rate (**Supplementary Table 4.2**). CDF_1 showed that when individuals formed new partnerships, their canonical score mean increased (**Supplementary Table 4.3, Figure 4.5 a, b** canonical score plots). This means that when individuals formed new partnerships, they mostly displayed higher within-pair agonism, and to a lesser extent from altered coordinated swimming (in *C. lunulatus lower* coordinated swimming; in *C. baronessa higher* coordinated swimming), higher agonism per competitor, and lower feeding bites than when they were in their enduring partnership (**Figure 4.5 a, b** canonical structure plots).

4.4.3.2 Recovery with new partnership endurance

Once re-paired, most focal individuals maintained association with their new partner throughout the remainder of the study (for *C. lunulatus,* six more days; for *C. baronessa,* eight more days), except for one *C. lunulatus* individual, who underwent a second re-pairing three days after its original partner was removed. As new pairs endured, focal individuals' activity profiles significantly changed (MANOVA: (*C. lunulatus*: Pillai's trace = 0.22, df = 1, $p < 0.001$; *C. baronessa*: Pillai's trace = 0.23, df = 1, $p < 0.001$). CDA of differences in activity profiles between days revealed that this change was mostly attributed to a reduction in intra-pair agonism, and to a lesser extent to altered coordinated swimming (in *C. lunulatus: increased*; in *C. baronessa: decreased*), reduced agonism per competitor, and increased feeding strikes. Notably, while these behaviors gradually recovered to original pairing levels, several days of cohabitation were needed for this to occur. Specifically, for both species, the standardized canonical coefficients of CDF_1 was mostly influenced by high within-pair agonism, and to a lesser extent by altered coordinated swimming (for *C. lunulatus less* coordinated swimming; for *C. baronessa more* coordinated swimming), high agonism per competitor and low feeding bite rate (**Supplementary Table 4.2**). CDF_1 showed that the increase in canonical score means invoked by re-pairing steadily recovered to pre-repairing levels after 4 days (**Supplementary Table 4.3, Figure 4.5 c, d** canonical score plots). This means that the higher levels of within-pair agonism, and altered coordinated swimming, higher agonism per competitor, and lower feeding bite rate invoked by re-pairing eventually abated after the course of 4 consecutive days (**Figure 4.5 c, d** canonical structure plots).

Overall the results from the partner replacement study suggest that re-pairing is costly to fishes, such that they suffer most from increased intra-pair conflict, and to a lesser extent from reduced pair-wise assistance during territory defence (represented by pair coordination), resulting in having to shift investment from feeding to territory defence. Importantly, these losses can be recovered by pair endurance.

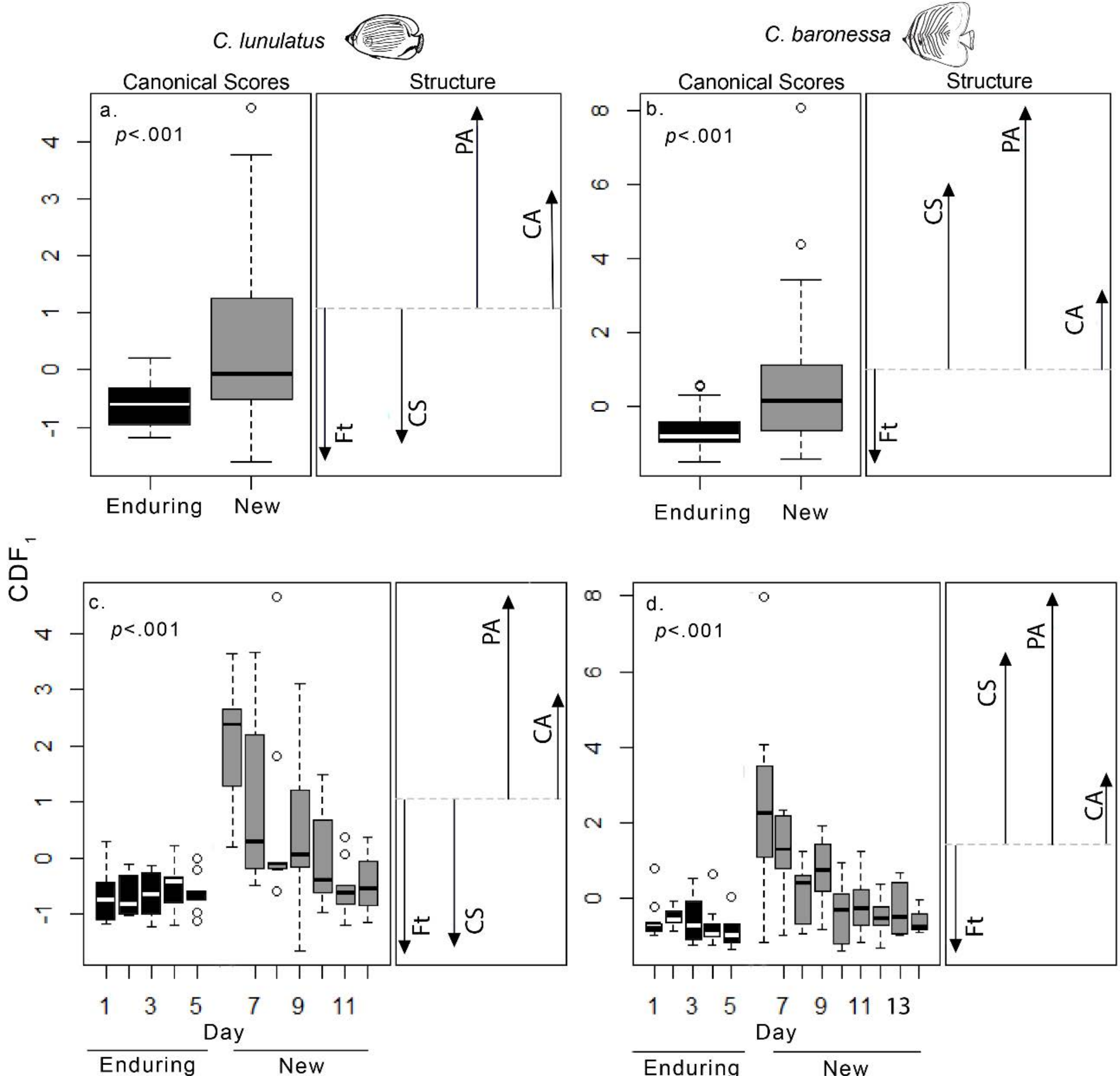


Figure 4.5. Changes in intra-pair relations, agonism towards competitors, and feeding strikes in response to re-pairing (a, b) and subsequent pair endurance (c, d). Means of standardized canonical scores of the first canonical discriminant function (CDF_1) are represented by box and whisker plots. The box delineates the first and third quartiles, the horizontal line shows the median, the whiskers indicate the maximum and minimum values, and circles represent outliers. Structure vectors show the relative strength (length of the vector relative to length of other vectors) and direction (+ or -) of the correlation between each contributing response variable and the canonical discriminant function. MANOVA p-value for change in activity profile in response to relationship phase or day is shown in the corner. In both species, re-pairing with a new partner increases intra-pair agonism (PA) (**a, b**). Concurrently, it reduces coordinated swimming (CS) in *C. lunulatus* (n = 9) (**a**), and increases coordinated swimming in *C. baronessa* (n=10) (**b**). These changes in intra-pair relations are associated with increased competitor antagonism (CA) and a reduction in total feeding strikes (Ft) (**a, b**). However, as new pairs endure, intra-pair relations recover along with recovered losses in competitor agonism and feeding efficiency (**c, d**).

4.4.4 Solitary vs. newly paired vs. enduringly paired individuals: Differences in liver hepatocyte vacuolation

For both *C. baronessa* and *C. lunulatus*, liver hepatocyte vacuole density varied significantly with social condition (*C. lunulatus*: Kruskal-Wallis = 19.39, df = 2, $p < 0.001$; *C. baronessa*: Kruskal-Wallis = 10.27, df = 2, $p = 0.006$). While there was no difference in liver vacuole density between pairs that were enduring and pairs that were relatively new (i.e., that persisted for 5-7 days), paired individuals had much greater hepatocyte vaculation than solitary counterparts (**Figure 4.6 a, b**). This indicates that pairing, but not pair endurance, is linked to improved physiological condition.

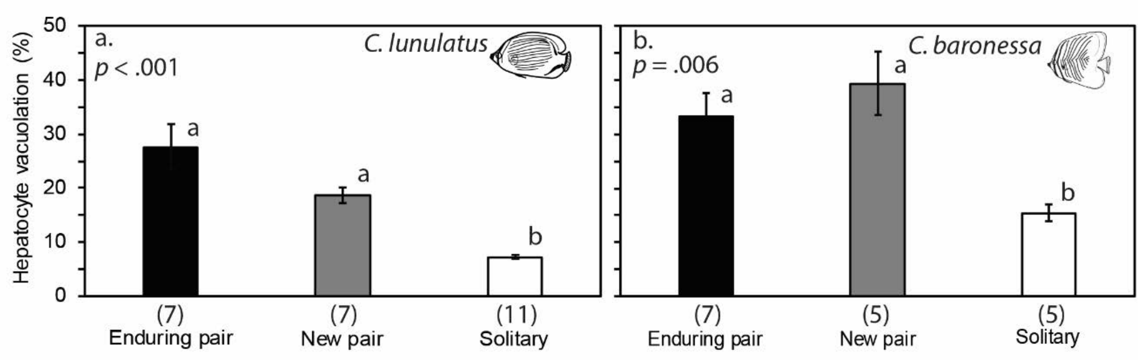


Figure 4.6. Variation in liver hepatocyte vacuole density among enduring pairs, new pairs (5-7-day persistence), and solitary individuals of *C. lunulatus* (a) and *C. baronessa* (b). Data are represented as the mean ± SEM. Kruskal-Wallis *p* value is shown in top corners, while groups not sharing the same letter are significantly different [Tukey and Kramer (Nemenyi) post-hoc analysis at $p < 0.05$]. Sample sizes are listed below each treatment group.

4.5 Discussion

4.5.1 Resource defense hypothesis for pairing

This study shows that paired individuals of both *C. baroness* and *C. lunulatus* exhibit cooperative territory defense, thereby corroborating previous studies that suggest that pair bonding in butterfyfishes is at least, in part, motivated by resource defense (Fricke, 1986; Hourigan, 1987, 1989; Tricas, 1989). *C. lunulatus* and *C. baronessa* pairs held permanent feeding territories that they defended against neighboring butterflyfishes, as previously reported in pairing butterflyfishes (Reese, 1975; Sutton, 1985; Fricke, 1986; Hourigan, 1987; Tricas, 1989; Yabuta and Berumen, 2014). However, the mode of territory defense assistance appeared to be species specific.

It has previously been proposed that, where assisted resource defense drives pairing, males nonetheless take-on the greatest burden for defense (Fuentes, 2002). However, my findings for *C. lunulatus* contributes to a growing body of literature indicating that males and females may contribute equally to resource defense, because both may equally benefit from each other's assistance (**Table 4.1**). Partners continuously swam within close proximity (2 metres), and frequently with coordinated movement (~ 56 % of the time) while foraging throughout their territory. When encountering neighboring butterflyfishes, territorial behavior was generally infrequent and passive, consistent with other butterflyfishes (Tricas, 1989; Roberts and Ormond, 1992). Conspicuous "pair swimming" characterized by very close proximity and coordinated movement has been previously reported in this species (Reese, 1975; Yabuta, 2002). Presumably, the function of pair swimming in butterflyfish pair bonds may be akin to duetting in bird pair bonds, such that it conspicuously advertises territory occupancy, thereby avoiding usurpation attempts by neighbors (Fricke, 1986). Notably, when territorial agonism did occur, it was mutually exerted by both partners. For both sexes, this ostensive co-defense appeared to provide an advantage to paired individuals over their solitary counterparts by improving their consumption level by ~ 58 %, and their energy reserves by 69 %, as indicated by feeding rates and hepatocyte vacuolation, respectively. Similarly, for *C. chrysurus* (= *paucifasciatus*), male-female partners continuously travel closely together throughout their territory, jointly engaging in territory defense, and both partners benefit by having higher feeding rates (Fricke, 1986).

In contrast to *C. lunulatus*, territory defense by pairs of *C. baronessa* appeared to be male-prioritized. Partners frequently traveled independently from each other, spending only ~10 % of their time coordinatedly swimming. Males patrolled larger areas within and along the boundaries of territories, exerting ~ 42 % more agonism towards neighboring butterflyfishes than females. This seemed to allow females to focus on foraging, notably within a more restricted area within the territory that contained a dominant assemblage of preferred coral (e.g., *A. hyacinthus* or *A. florida*). Consequently, paired females appeared to consume 43% more total, and 96% more preferred coral then their solitary counterparts. Moreover, and probably as a consequence of increased food intake, liver lipid reserves in paired individuals were higher by ~ 57 % than solitary individuals, though no distinction was made between males versus females. Among species that appear to pair for assisted resource defense purposes, male-prioritized defense is the most commonly reported mode of assistance (**Table 4.1**), where it has been previously attributed to supporting female food consumption in pair bonding birds (Black, 2001; Black et al., 2014) and butterflyfishes (Hourigan, 1989). In some geese, males act as sentinels, fending off competitors from nesting and feeding sites while females spend the majority of their time feeding (Boyd, 1953; Raveling, 1970; Black and Owen, 1988, 1989; Sedinger and Raveling, 1990; Forslund, 1993; Black et al., 2001). Similarly, in the congeners *C. multicinctus* and *C. quadrimaculatus,* males take-on the

majority of feeding territory defense duty, allowing females to spend more time feeding (Hourigan, 1987; Tricas, 1989). This form of sexual division of labor is thought to occur when female egg production is especially costly, and disproportionally more male assistance is required for females to build the energy reserves needed for egg production (Hourigan 1989; Lamprecht, 1989). Egg production in female *C. baronessa* may be particularly energetically costly, thereby favoring male-prioritized defense. *Chaetodon baronessa's* preferred diet, *Acropora* corals provide the best energetic return among coral families, after accounting for feeding efficiency (Graham, 2007; Cole et al., 2011). However, Cole et al. (2011) found that *C. baronessa* exhibit higher feeding rates and subsequently consume more coral tissue per day than *C. lunulatus* and other congeners, indicating that perhaps they have a relatively low energetic absorption efficiency. In order to ascertain this possibility, analysis of energetic absorption efficiency (relative to other corallivorous species) (e.g., Hourigan, 1987, 1989)) would be required. Interestingly, paired *C. baronessa* males appeared to consume 49 % more total, and 96 % more preferred coral than their solitary counterparts. This might suggest that although males receive relatively little territorial defense assistance from females, it may be sufficient to confer an advantage to food consumption.

In previous studies of butterflyfishes, cooperative territory defense led to increased feeding rates by reducing the time needed for territory defense, thereby freeing up time to invest in feeding (Fricke, 1986; Hourigan, 1987). In this study, however, I found no evidence that paired individuals could effectively defend territories with lower levels of per capita agonism relative to solitary counterparts. Importantly, rates of agonism tended to be highly variable and sample sizes were relatively low, limiting the power to detect differences in rates of agonism between paired and solitary individuals. It is also possible that pair bonding and cooperative resource defense enabled paired butterflyfishes to establish territories in areas with greater food availability, thereby providing fitness benefits even though rates of agonism were not different. In support of this idea, territories of paired fishes did have higher coral cover, and disproportionate amounts of preferred corals, relative to territories of solitary butterflyfishes. My study also showed that paired butterflyfishes had higher feeding rates and improved physiological condition (as evident based on comparisons of hepatocyte vacuolation) compared to solitary counterparts. However, increased feeding may be a consequences of pairing *per se* (e.g., increased predator vigilance) rather than necessarily tied to assisted territory defense. However, previous experiments showing i) evidence for assisted territory defense among pairs, and ii) marked declines in these attributes following partner removal (energy storage notwithstanding) provide empirical support for assisted resource defense being the causal factor in these relationships (Fricke, 1986; Hourigan, 1987).

If corallivorous butterflyfishes pair for resource defense assistance in order to maximize feeding investment, then why don't they exhibit even larger group sizes? Perhaps this is because beyond a group size of two, the costs of group living within a fixed territory size may exceed the benefits of assistance (Fuentes, 2002), and expanding territory size is uneconomical (Tricas, 1989), setting the optimal group size to two members. Moreover, while pairing, heterosexual partnerships are favored, where intersexual agonism and mate guarding may further limit additional group membership (Hourigan, 1989).

4.5.2 Functional contribution of partner fidelity

Ecological reasons for why species who pair for assisted resource defense display long-term partner fidelity are almost wholly unknown. In the current study, I showed that there are definite benefits associated with mate familiarity, which comes from endurance of pair bonds. Experimentally inducing new partnerships caused an immediate and marked decline in partner relations. This was primarily driven by increased in intra-pair conflict, made apparent by heightened levels of fleeing, chasing, and circling between partners despite persistence of the new partner; and to a lesser extent by reduced expression of species-specific modes of assisted territory defence, as indicated by decreased pair swimming in *C. lunulatus*, and increased pair swimming in *C. baronessa*. This decline in partner relations attracted neighbouring pairs, who in response initiated and engaged in heightened territorial activity with the new pair. Subsequently, newly paired individuals suffered from having to shift investment from feeding to territory defence, as indicated by associated increases in agonism towards neighbouring competitors and declines in feeding strikes. However, as new partnerships subsequently endured, these intra- and inter-pair disruptions abated, and incurred costs to individual territory defence-feeding budgets recovered. Similar to my findings, it has been shown in other species who pair bond for assisted resource defense that widowed individuals with established territories will initially aggressively resist the elicitation to form new partnerships prior to conceding (wood louse, *Hemilepistus reaumuri*, Eduard and Linsenmair, 1971). It has also been shown that pair bonds of longer duration monopolize higher quality feeding territories, ostensibly through enhanced co-operation, and this is further linked to improvements in life-time reproductive success (barnacle geese, *Branta leucopsis*: Black, 2001; Black et al., 2014). My results suggest that partner fidelity plays a critical role in contributing towards assisted resource defence in chaetodontid species, and inhibits intra-pair conflict, ultimately conferring gains in feeding investment. Although I found no evidence that this translates into energy reserve gains, this may have been a result of sampling after new pairs had already endured for ~ 1 week, and displayed fully-recovered behavioral profiles.

How and why might partner fidelity promote assisted resource defense and inhibit intra-pair conflict in these species? Perhaps partner fidelity improves assisted territory defense through partner familiarity. Indeed, it has been shown in fishes that cooperation with specific partners stabilizes over time, because individuals are more cooperative with familiar partners (Granroth-Wilding and Magurran, 2013). The underpinning mechanism(s) for this may be unique to the species-specific mode of cooperative assistance. For *C. lunulatus*, who appears to work together simultaneously to provide mutual assistance, partner familiarity may facilitate learning and accurate prediction of partner behavior (e.g., chosen defense route or routine), thereby fine-tuning pair-wise coordination (Chivers et al., 1995; Griggio and Hoi, 2011; Sanchez-Macouzet et al., 2014; Leu et al., 2015). For *C. baronessa*, who appears to exhibit male-prioritized assistance in exchange for sequentially reciprocated partitioning of services/resources by females (i.e., direct reciprocity), partner familiarity may allow individuals to learn which "partner control mechanism" is best suited to stabilize cooperation, based on the tendency of partners to reciprocate (or cheat) in the past (Gomes et al., 2009; Wubs et al., 2016). Upon new pair formation, partner familiarity (and therefore effective co-operation, and co-operatively derived feeding benefits) takes several days to develop; however, the cost of food sharing is immediately incurred. Hence, until co-operative relations develop, the costs (food sharing) outweigh the benefits (maximizing feeding investment) of pairing, causing territory holders to agonistically resist their new partner. In addition to promoting intra-pair relations, there may be several other ecological reasons for long-term partner fidelity in pair bonding chaetodontids. For example, partners may experience a delay in the time at which their services are reciprocated (Whiteman and Côté, 2004), or mutual site-attachment to the feeding territory, airising if it new territories are scarce or competitively costly to acquire (Tricas, 1989; see 'dear enemy' phenomenon, Wilson, 2000).

4.5.3 Conclusions

Energy acquisition is fundamental to growth, reproduction, and maintenance (Hughes, 1997). However, corallivorous butterflyfishes rely almost exclusively on a diet of hard coral (Pratchett, 2005), which is a relatively nutrient poor, but abundant resource (Tricas, 1989b). Consequently, both sexes are energy maximizers, feeding almost continuously (Tricas, 1989a). Foraging is constrained by time spent on other activities, including territory defense. As such, attributes that alleviate time constraints on foraging, are likely to directly benefit individual fitness (Hourigan, 1989). This study corroborates with previous studies on butterflyfishes, suggesting that partners of *C. lunulatus* and *C. baronessa* pairs display territorial defense assistance, increasing their consumption of coral food and subsequently energy reserves, relative to solitary counterparts. I further show evidence that partner fidelity plays a critical role in this function by inhibiting conflict and promoting territorial defense assistance between

partners, providing an ecological benefit for pair formation and fidelity in these species. Whether this translates into an adaptive advantage should now be addressed by undertaking long-term monitoring studies to discern whether enduring pair bonding also confers relatively higher survivorship and/or life-time fitness benefits (Black, 2001).

Chapter 5: General discussion

Pair bonding has independently evolved in all major vertebrate lineages (Reichard and Boesch, 2003), where it represents a major defining feature of species-specific social structure (Goodson and Kingsbury, 2011), including that of humans' (Quinlan and Quinlan, 2007; Quinlan, 2008). While proximate reasons for *how* pair bonding occurs (i.e., it underlying neurobiology), and ecological reasons for *why* pair bonding occurs (i.e., its adaptive functions(s)) have become well established among more derived vertebrates (i.e., mammals and birds) (Young and Wang, 2004; Lukas and Clutton-Brock, 2013; Reichard and Boesch, 2003; Freeman and Young, 2013; Opie et al., 2013; Donaldson and Young, 2016), considerably less is known about the neural or adaptive basis of pair bonding in earlier vertebrates (i.e., reptiles, amphibians, and fishes). Understanding pair bonding among these lineages is important, not only because it generates insight into their own nature, but also because it is fundamental in understanding the evolutionary history of pair bonding. Moreover, inter- and intra-specific variation in pairing among birds and mammals is confounded with several other attributes, most notably bi-parental care (Goodson et al., 2006; Goodson and Kingsbury, 2011). In contrast, many pair bonding earlier vertebrates have no parental care, enabling independent analyses of the proximal and ultimate basis of pairing.

Among teleost fishes, there are 394 pair bonding species spanning 36 families (Whiteman and Côté, 2004; Brandl and Bellwood, 2014). These families also display variation in sociality among species/individuals that are both phylogenetically and geographically close (Hourigan, 1989; Oliveira, 2012, Brandl and Bellwood, 2014), providing foremost opportunities for research into pair bonding. In addition, as extant members of ray-finned fishes (actinopterygians), whose origins date back to ~422 MYA (Silurian period) (Benton and Donoghue, 2007), teleosts generate insight into the earliest origins of vertebrate pair bonding. Coral reef butterflyfishes have 77 species of pair bonding fishes (**Table 1.2**), accounting for 20% of all pair bonding marine fishes. This well established natural history (Cole and Pratchett, 2014; Kulbicki et al., 2014; Pratchett, 2014; Yabuta and Berumen, 2014), along with spectacular variation in sociality among sympatric individuals and species (Reese, 1975; Yabuta and Berumen, 2014), and general amenability to aquaria (Belbeek, 2014), makes the butterflyfishes an exceptional model system for comparatively and experimentally exploring the physiological and adaptive basis of pair bonding in teleosts (**Chapter 2**).

5.1 Butterflyfishes as a model system for teleost pair bonding

In situ observations of sympatric butterflyfishes at Lizard Island, in the northern Great Barrier Reef (GBR), revealed strong intra- and inter-specific variation in sociality (**Chapter 2**). Specifically, 84% of *C. lunulatus,* 78% of *C. baronessa,* and 71% of *C. vagabundus*

adults were pair bonded, whereas 88% of *C. rainfordi,* 90% of *C. plebeius,* and 80% of *C. trifascialis* were solitary. While pairing sociality varies markedly on these levels of social organization, several other key attributes, including parental care, territoriality, feeding ecology, environmental conditions, and relatedness do not co-vary (**Table 2.2**). However, the mating system is assumed to co-vary with pairing sociality in these species, representing a potential confound and therefore warranting the development of an experimental assay that can provide functional validation. **Chapter 2** then showed that in a classic laboratory assay, the "two-choice proximity" assay (a.k.a. "partner preference" assay) (Williams et al., 1992; Adkins-Regan, 2016), a routinely used behavioral proxy for pair bonding, "partner preference" (Williams et al., 1992; Young et al., 2011) was reliably elicited in males of one strongly pair bonding species, *C. lunulatus*. When given a choice to affiliate with either their partner or a non-partner conspecific, males spent on average 54/60min affiliating with their partner, and only 8/60min affiliating with a non-partner. Taken together, these findings reaffirm previous classification of the sociality of these species (Reese, 1975; Yabuta and Berumen, 2014). They furthermore validate that the proposed butterflyfish systems are amenable for undertaking highly controlled comparative, and reliable experimental research into fish pair boding.

5.2 Neural basis of pair bonding in butterflyfishes

Much of our understanding of pair bonding neurobiology stems from extensive studies on few mammalian model systems, primarily *Microtus* voles (Carter et al., 1995; Young, 2003; Aragona and Wang, 2004; Young and Wang, 2004; McGraw and Young, 2010; Young et al., 2011; Freeman and Young, 2013; Johnson and Young, 2015; Gobrogge and Wang, 2016). Complimentary use of comparative and experimental (pharmacological) studies has provided significant insight into the neural circuitry underlying pair bonding in *Microtus* voles (ibid), comprising four integral neurochemical systems: the oxytocin (OT), arginine vasopressin (AVP), dopamine (DA), and opioid (OP) systems (Johnson and Young, 2015; Donaldson and Young, 2016). In order to promote partner attachment, OT and AVP nonapeptides appear to mediate social memory by acting on the prefrontal cortex (PFC), striatal nucleus accumbens (NAcc), and on the NAcc, lateral septum (LS) and ventral pallidum (VP) and the anterior hypothalamus (antHYP), respectively (Winslow et al., 1993; Young et al., 1997; Cho et al., 1999; Young et al., 2001; Liu and Wang, 2003; Gobrogge et al., 2007, 2009; Numan and Young, 2016). Whereas, dopamine appears to mediate partner reward learning and maintenance by targeting the striatal NAcc (Gingrich et al., 2000; Aragona et al., 2003, 2006; Resendez et al., 2016). Finally, OP-mu-opioid receptor (MOR) signaling presumably mediates positive hedonics involved in partner reward learning during pair formation, and targeted brain regions include sub-structures of the striatum, including the caudate putamen (CP), dorsal striatum, and NAcc (Burkett et al., 2011; Resendez et al., 2012, 2013, 2016).

This study provides evidence that the neurochemical and –anatomical substrates that govern *C. lunulatus* pair bonding are very similar to those of the mammalian model, *M. ochrogaster* (**Chapter 3**). More specifically, I found that in males, pharmacologically blocking isotocin (IT, teleost homologue of OT) and arginine vasotocin (AVT, teleost homologue of AVP) V1a receptors attenuates selective affiliation with an established female partner; whereas blocking dopamine D1 and MOR receptors has no significant effect. Comparisons of ITR, V1aR, D1R, D2R, and MOR gene expression within eight brain regions between pair bonded and solitary individuals showed that in females, differences in IT and AVT V1a nonapeptide receptor expression within the lateral septum-like region (the ventral and lateral regions of the ventral telencephalon, Vv/Vl) are associated with differences in pairing phenotype. It further revealed that in both sexes, differences in dopamine D1R, D2R, and MOR gene expression within several regions of the mesolimbic reward system, including the striatum, are associated with differences in pairing phenotype.

Results of pharmacological studies in *C. lunulatus* (**Chapter 3**) corroborate findings of recent studies showing that IT and AVT systems play a fundamental role in pair bonding in fishes (Oldfield and Hofmann, 2011; but see O'Connell et al., 2012), much like OT and AVP in later vertebrates. Moreover, this study provides the first evidence for the involvement of dopamine and opioid systems, and specific brain regions, in pair bonding in fishes. Historically, it has been assumed that the convergence of evolutionarily labile social behaviors, especially across distinct lineages, are based on entirely different regulatory processes (Butler and Hodos, 2005). For instance, theoretically, many different pre-existing neural mechanisms could be modified to promote pairing behavior (Goodson and Thompson, 2010). Conversely, evolution may follow similar trajectories in different lineages due to canalization-like effects (Goodson and Thompson, 2010). Indeed, the recent discoveries that the neuro-chemical and –anatomical components pair bonding are highly conserved across vertebrates (Archer and Chauvet, 1995; Pombal et al., 2007; Dreborg et al., 2008; Sundström et al., 2010; O'Connell and Hofmann, 2011, 2012; Garrison et al., 2012; Robertson et al., 2012; Ericsson et al., 2013) makes exploring the possibility of convergent regulatory mechanisms a timely endeavor (Fink et al., 2006; Goodson and Thompson, 2010; Turner et al., 2010; Goodson and Kingsbury, 2011). These findings shed new light onto the evolutionary history of regulatory mechanisms for pair bonding, tentatively suggesting that at least in selective cases, the convergence of pair bonding across exceptionally distinct lineages (i.e., mammals and fishes, which are separated by ~450 million years of independent evolution) is a product of at least partially-convergent underlying neural mechanisms.

5.3 Ecological basis of pair bonding in Chaetodontidae

Extensive research, primarily on birds, humans, and other mammals, suggests that pair bonding is attributed to three key reproductively-based adaptive functions: the benefits

of mate choice, of mate-guarding, and of bi-parental care (Reichard and Boesch, 2003). However, the (albeit relatively uncommon) occurrence of pair bonding between homosexual (Gore, 1983; Tricas, 1986; Bagemihl, 1999; Pratchett et al., 2006; Young et al., 2008; Elie et al., 2011; Brandl and Bellwood, 2013), sexually immature (Fricke, 1986, Tricas, 1986; Pratchett et al., 2006), and non-parental (Fricke, 1983; Kokita and Nakazono, 2001) partners strongly suggests that pair bonding may serve alternative or additional functions(s) in at least some species (Pratchett et al., 2006). One notable example is pair bonding coral-feeding butterflyfishes, because they display all of these peculiarities (Gore, 1983; Fricke, 1986, Tricas, 1986; Pratchett et al., 2006). In 1975, E.O Wilson put forth an alternative explanation for pair bonding that has since garnered relatively less attention. He proposed that pair bonding may arise when an organism's territory contains such a valuable resource that two individuals are required to defend it. In this context, resources may be valuable due to being highly depended upon, yet scarce or of low quality (Wilson, 2000; Rutberg, 1983). This *assisted resource defense hypothesis* (ARDH) for pairing (Tecot et al., 2016) puts forth several predictions (see: Rutberg, 1983; Mathews, 2002; Whiteman and Côté, 2004; Tecot et al., 2016), including (but not limited to): 1) males predominantly defend resources (Rutberg, 1983; Whiteman and Côté, 2004; Tecot et al., 2016), benefitting females (Whiteman and Côté, 2004), or 2) both partners mutually defend resources (Rutberg, 1983; Mathews, 2002; Whiteman and Côté, 2004; Tecot et al., 2016), benefitting both partners (Mathews, 2002; Whiteman and Côté, 2004). Empirical tests of these predictions and *in situ* observations have supported the ARDH for pair bonding in a growing range of taxa (Vaughan and Vaughan, 1986; Morley and Balshine, 2002; Kotrschal et al., 2006; Rosell and Thomsen, 2006; Elie et al, 2011; Black et al., 2014; Illes, 2015; Tecot et al., 2016), but the ARDH has rarely been tested in coral-feeding butterflyfishes: *C. paucifasciatus* (Fricke, 1986), *C. multicinctus,* and *C. quadrimaculatus* (Hourigan, 1987, 1989). Furthermore, it remains almost wholly unknown why species who pair bond for assisted resource defense purposes display long-term partner fidelity? One hypothesis is that pairs might endure because it improves defense assistance, thereby improving resource acquisition/maintenance; however, this has only been tested in a single species of bird, barnacle geese, *Branta leucopsis* (Black, 2001, Black et al., 2014).

In at least two species of butterflyfishes, *C. lunulatus* and *C. baronessa*, pairs display cooperative territory defense (**Chapter 4**), which may provide a significant benefit that promotes both the formation and endurance of pair bonds. In *C. lunulatus,* both partners frequently display conspicuous proximate and coordinated swimming (i.e., "pair swimming") and mutually engage in territory defense. In association with mutual defense assistance, both partners appear to show marked improvements in feeding (indicated by feeding strikes) relative to solitary conspecifics. Partners also appear to have improved energy reserves (indicated by liver hepatocyte density) relative to solitary counterparts. In *C. baronessa,* by contrast, partners spend little time pair swimming, and males disproportionately contribute to territory defense. This male-

prioritized territorial defense in *C. baronessa* does nonetheless, appear to result in higher rates of feeding and corresponding increases in the physiological condition of paired versus solitary fishes. These findings contribute to the relatively few but growing number of studies that support the ARDH for pair bonding in coral-feeding butterflyfishes and in vertebrates more broadly. These results, taken together with occurrence of paring among reproductively immature or homosexual partners (Gore, 1983; Fricke, 1986, Tricas, 1986; Pratchett et al., 2006) demonstrate that not only might assisted resource defense contribute to the adaptive function of pair bonding in butterflyfishes, but that it might in fact be more influential to the occurrence of pair bonding than reproductive benefits.

Assisted resource defense provides a non-reproductive (ecological) basis for pair bonding across a broad range of distinct lineages, including fishes (Fricke, 1986; Hourigan, 1987, 1989; Morley and Balshine, 2002, 2003, current study), birds (Kotrschal et al., 2006; Elie et al., 2011), Black, 2001; Black et al., 2014; Illes, 2015), and mammals (Vaughan and Vaughan, 1986; Rosell and Thomsen, 2006; Tecot et al., 2016), suggesting that it may have contributed to the early evolution and subsequent wide-spread convergence of vertebrate pair bonding. It is also clear that enduring pair bonds further increase the effectiveness of cooperative ecological functions. While coral feeding butterflyfish will readily pair with new individuals following mate loss, newly formed pairs exhibited high levels of intra-pair agonism, corresponding with lower rates of feeding and increased territory defense (**Chapter 4**). However, newly formed pairs did exhibit steady declines in agonism with corresponding improvements in feeding and territory defense within 6-8 days. These findings provide some of the first insight into why species who pair for assisted resource defense purposes display long-term partner fidelity: through ecological benefits from improved partner relations (Black, 2001; Black et al., 2014).

Pair bond explanations: butterflyfishes
(Current form: the trait at one point of time)

How it works
Mechanism
(The structure of the trait)
Neural systems
IT OP DA AVT

What it is for
Ecological function
(How variations in trait interact with environment to influence organism)
Assisted resource defense

Figure 5.1. Complimentary biological explanations for how and why pair bonding occurs among *Chaetodon spp*. Explanations apply to the trait in its current form, rather than historical/ontogenetic sequences that resulted in the trait (Tinbergen, 1963; Nesse, 2013). **Mechanism (how):** nonapeptide (isotocin (IT), arginine vasotocin (AVT), dopamine (DA), and opioid (OP) systems mediate pair bonding behavior by acting within nodes of the vertebrate social decision making network. **Ecological function (why):** Pair bonding functions to maximize food resource consumption by promoting assisted food resource defense between partners. Pictures featured are model organisms used for exploring the mechanistic basis (*C. lunulatus*), and ecological basis (*C. lunulatus* and *C. baronessa*). Brain illustration adapted from Dewan and Tricas, 2014, with permission.

5.4 Conclusions

In summary, this thesis addresses neural explanations for *how* and ecological reasons for *why* pair bonding occurs in teleosts, using butterflyfish as an effective model system. Nonapeptide, dopamine, and opioid systems acting within specific nodes of the vertebrate social decision making network facilitate pair bonding, to provide social assistance during defense of food resources (**Figure 5.1**). The repeated evolution of pair bonding across vertebrates is undoubtedly a consequence of the trait serving several adaptive functions (Reichard and Boesch, 2013), and it is theoretically possible that this has occurred through the modification of many different neural mechanisms (Goodson and Thompson, 2010). However, these findings, when compared to pre-existing findings in mammals and birds, tentatively suggest that at least in selective cases, the convergence of vertebrate pair bonding among distant lineages has been facilitated by coopting homologous neural structures to facilitate an analogous ecological function. In order to determine the extent to which this has occurred, complementary studies across a wider range of vertebrates (most urgently amphibians and reptiles) are now needed.

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Appendix A

Supplementary material

Chapter 3

Table 3.1. Brain region punching regime standardized across individuals.

	Brain region	Total # of punches (# punches/section)	Section #s on which regional punches were made (rostral to caudal sequence)
1	Dorsal part of the ventral telencephalon (Vd)	4 (2)	Section # 3-4 of telencephalon
2	Medial part of the dorsal telencephalon (Dm)	14 (2)	Section # 3-9 of telencephalon
3	Lateral part of the dorsal telencephalon (Dl)	14 (2)	Section # 3-9 of telencephalon
4	Supracommissural part of the ventral telencephalon (Vs)	6 (1)	Section # 4-9 of telencephalon
5	Central part of the ventral telencephalon (Vc)	4 (2)	Section # 9-10 of telencephalon
6	Preoptic area (POA)	12 (1)	Section # 9-20 of telencephalon
7	Posterior tuberculum (TPp)	3 (1)	Section # 3-5 of diencephalon
8	Ventral/lateral portion of the ventral telencephalon (Vv/Vl)	12 (2)	Section #3-8 of telencephalon

Table 3.2. Primers used for cloning, gene-specific reverse transcription, and qPCR are listed for target genes (ITR, V1aR, D1R, D2R, MOR) and reference gene (18S ribosomal) RNA.

Gene	Cloning primers	Reverse transcription primers	qPCR primers
ITR	Pair a (using *C.lunulatus* transcriptome): F: 5'-TTTTGTGCAGGTTGGTGAAA R:5'-AGATCCAGGGGTTACAGCAG Pair b (to obtain exon junction): F: 5'-TTTTGTGCAGGTTGGTGAAA	R:5'-GGCTGCTCGTGCTTTTAATG	F:5'-GTCTGTTGGACCCCCTTTTT R:5'-CAGCAGCATGGAGATGATGA

	R: 5'- GAATTGAACCGCTGGATGTT		
V1aR	Pair a (using *C.lunulatus* transcriptome): F: 5'- GGAAGACGATGACTGGTGCT R: 5'- AGCTGTTGAGACTGGCAAGG Pair b (to obtain exon junction): F: 5'- GGAAGACGATGACTGGTGCT R: 5'- TGTTAGACCTCCTGGCTGCT	R:5'-AGGGCTTCGATTGGTCATCT	F:5'- CTGTGTGGGATGAAAACTTCCT R:5'- AGGAGGTGACCGCTGAAGAT
D1R	Pair a (using *C.lunulatus* transcriptome): F: 5'- GAACGCAAGATGACCCCTAA R: 5'- CCTGTCAGGCATGTCCTTTT	R:5'-TCAAAGGTGGAGCTGAT	F:5'- TCGAACATGGAGAGTGAGAGC R:5- CCAGCAGCACACAAACACTC
D2R	Pair a (using *C.lunulatus* transcriptome): F: 5'- TTGCTGTAAGCTGCCATTTG R: 5'- TTTTGCCTGAAACAGGTCA	R:5'-TCTGTTGCAGGATCTCCATTC	F:5'-AACGGGAGCTTTCCTGTCA R:5'- GCTGTTGTTCAGCTCATCCAG
MOR	Pair a (using *C.lunulatus* transcriptome): F: 5'- AGACCGCCACCAACATCTAC R: 5'- GGATGAGGGTTACGACAGGA	R:5'- CGCAGGTTCCTGTCCTTCT	F:5'- TCATGTTCATGGCCTCCAC R:5'- GCAGATCTTCAGCAGGGTGT
18S	Pair a (using *C.lunulatus* transcriptome): F: 5'- GAGACTCCGGCATGCTAACT R: 5'- GTAATGATCCTTCCGCAGGT	5'- ATAGTCAAGTTTGATCGTCTTCTCG	F:5'- CAGTAAGCGCGGGTCATAAG R:5'- CGATCCGAGGACCTCACTAA

Table 3.3 Optimized qPCR thermal cycling parameters for each gene.

Gene	Enzyme mix	Cycle function	# cycles	Temp. (˚C)	Time

D2R	PerfeCTa ® SYBR ® Green SuperMix	Initial denature	1	95	3 min
		Denature	45	95	15 sec
		Primer anneal + extension + plate read		60	1 min
		Melt curve			
ITR V1aR MOR D1R	PerfeCTa ® SYBR ® Green FastMix	Initial denature	1	95	30 sec
		Denature	45	95	5 sec
		Primer anneal		55	15 sec
		Extension + plate read		70	10 sec
		Melt curve			

Table 3.4. ANOVA table for ITR, V1aR, D1R, D2R and MOR gene expression in brain regions of *C. lunulatus* compared between 4 combinations of sex (male and female) and social system (pair bonded and solitary).

Source	df	MS	F	*P*
ITR				
Dl				
Sex	1	0.0	0.0	1.0
Social system	1	0.0	0.0	1.0
Sex X Social System	1	0.0	0.0	1.0
Residual	12	1.011E-12		
Dm				
Sex	1	0.0	0.0	1.0
Social system	1	0.0	0.0	1.0
Sex X Social System	1	0.0	0.0	1.0
Residual	14	1.106E-13		
POA				
Sex	1	1.271E-10	3.752	.072
Social system	1	9.742E-11	2.875	.111
Sex X Social System	1	.000	.000	1.000
Residual	15	3.388E-11		
TPp				
Sex	1	.000	.000	1.000
Social system	1	.000	.000	1.000
Sex X Social System	1	.000	.000	1.000
Residual	14	4.071E-11		
Vc				
Sex	1	3.825E-10	1.945	.197
Social system	1	3.049E-10	1.550	.245
Sex X Social System	1	4.582E-10	2.330	.161
Residual	9	1.966E-10		
Vd				
Sex	1	.000	.	.
Social system	1	.000	.	.
Sex X Social System	1	.000	.	.
Residual	13	.000		

Vs				
Sex	1	.000	.000	1.000
Social system	1	.000	.000	1.000
Sex X Social System	1	.000	.000	1.000
Residual	15	6.369E-13		
Vv/Vl				
Sex	1	4.252E-9	5.705	.033*
Social system	1	4.329E-9	5.808	.031*
Sex X Social System	1	4.146E-9	5.563	.035*
Residual	13	7.453E-10		
V1aR				
Dl				
Sex	1	.000	.000	1.000
Social system	1	.000	.000	1.000
Sex X Social System	1	.000	.000	1.000
Residual	13	3.537E-13		
Dm				
Sex	1	.000	.000	1.000
Social system	1	.000	.000	1.000
Sex X Social System	1	.000	.000	1.000
Residual	14	2.595E-13		
POA				
Sex	1	2.857E-10	4.655	.048*
Social system	1	5.391E-11	.878	.364
Sex X Social System	1	1.201E-10	1.956	.182
Residual	15	6.138E-11		
TPp				
Sex	1	8.087E-10	2.211	.161
Social system	1	2.589E-11	.071	.794
Sex X Social System	1	8.843E-11	.242	.631
Residual	13	3.657E-10		
Vc				
Sex	1	8.087E-10	2.211	.161
Social system	1	2.589E-11	.071	.794

Sex X Social System	1	8.843E-11	.242	.631
Residual	13	3.657E-10		
Vd				
Sex	1	1.071E-10	2.523	.136
Social system	1	7.028E-11	1.656	.221
Sex X Social System	1	6.016E-11	1.417	.255
Residual	13	4.244E-11		
Vs				
Sex	1	4.075E-10	2.278	.152
Social system	1	5.321E-11	.297	.594
Sex X Social System	1	3.045E-11	.170	.686
Residual	15	1.789E-10		
Vv/Vl				
Sex	1	8.806E-7	5.221	.040*
Social system	1	1.066E-6	6.323	.026*
Sex X Social System	1	8.786E-7	5.209	.040*
Residual	13	1.687E-7		
D1R				
Dl				
Sex	1	9.245E-10	.064	.804
Social system	1	6.931E-8	4.797	.047*
Sex X Social System	1	8.438E-10	.058	.813
Residual	13	1.445E-8		
Dm				
Sex	1	1.032E-9	.331	.576
Social system	1	1.440E-8	4.619	.053
Sex X Social System	1	1.028E-9	.330	.576
Residual	12	3.118E-9		
POA				
Sex	1	8.807E-8	1.575	.230
Social system	1	8.145E-7	14.563	.002*
Sex X Social System	1	9.042E-8	1.617	.224
Residual	14	5.593E-8		

TPp				
Sex	1	1.366E-7	1.028	.334
Social system	1	3.105E-7	2.337	.157
Sex X Social System	1	1.324E-7	.997	.342
Residual	10	1.328E-7		
Vc				
Sex	1	1.636E-8	.106	.753
Social system	1	6.855E-7	4.425	.065
Sex X Social System	1	1.577E-8	.102	.757
Residual	9	1.549E-7		
Vd				
Sex	1	3.156E-8	1.005	.342
Social system	1	9.106E-8	2.899	.123
Sex X Social System	1	3.507E-8	1.117	.318
Residual	9	3.141E-8		
Vs				
Sex	1	3.223E-9	.313	.586
Social system	1	1.067E-7	10.344	.007*
Sex X Social System	1	2.954E-9	.286	.602
Residual	12	1.031E-8		
Vv/Vl				
Sex	1	2.400E-5	.305	.591
Social system	1	3.809E-5	.484	.500
Sex X Social System	1	3.539E-5	.450	.515
Residual	12	7.867E-5		
D2R				
Dl				
Sex	1	3.721E-8	1.300	.275
Social system	1	4.933E-7	17.230	.001*
Sex X Social System	1	3.717E-8	1.298	.275
Residual	13	2.863E-8		
Dm				
Sex	1	1.928E-9	.196	.665
Social system	1	1.577E-7	15.993	.001*

Sex X Social System	1	1.917E-9	.194	.666
Residual	15	9.858E-9		
POA				
Sex	1	2.543E-7	1.545	.232
Social system	1	4.464E-6	27.122	.000*
Sex X Social System	1	2.196E-7	1.334	.265
Residual	16	1.646E-7		
TPp				
Sex	1	1.823E-7	.448	.514
Social system	1	3.761E-6	9.242	.009*
Sex X Social System	1	2.351E-7	.578	.460
Residual	14	4.070E-7		
Vc				
Sex	1	7.765E-8	.475	.508
Social system	1	2.619E-6	16.024	.003*
Sex X Social System	1	1.073E-7	.657	.439
Residual	9	1.635E-7		
Vd				
Sex	1	5.411E-11	.000	.984
Social system	1	3.767E-7	3.041	.107
Sex X Social System	1	6.335E-11	.001	.982
Residual	12	1.238E-7		
Vs				
Sex	1	5.920E-8	2.058	.172
Social system	1	4.123E-7	14.332	.002*
Sex X Social System	1	6.116E-8	2.126	.165
Residual	15	2.877E-8		
Vv/Vl				
Sex	1	.001	.474	.503
Social system	1	.001	.560	.468
Sex X Social System	1	.001	.613	.448
Residual	13	.002		

MOR

Dl				
Sex	1	1.014E-8	.267	.614
Social system	1	2.345E-7	6.164	.026*
Sex X Social System	1	1.114E-8	.293	.597
Residual	14	3.804E-8		
Dm				
Sex	1	1.218E-8	.705	.414
Social system	1	2.790E-8	1.614	.223
Sex X Social System	1	1.203E-8	.696	.417
Residual	15	1.729E-8		
POA	1	3.786E-7	1.605	.226
Sex	1	5.086E-6	21.554	.000*
Social system	1	3.754E-7	1.591	.228
Sex X Social System	14	2.359E-7		
Residual				
TPp				
Sex	1	7.314E-8	.385	.545
Social system	1	2.079E-6	10.943	.005*
Sex X Social System	1	1.170E-7	.616	.446
Residual	14	1.900E-7		
Vc				
Sex	1	3.711E-7	.800	.394
Social system	1	2.176E-6	4.690	.059
Sex X Social System	1	3.672E-7	.791	.397
Residual	9	4.640E-7		
Vd				
Sex	1	8.618E-7	.352	.564
Social system	1	3.226E-6	1.319	.273
Sex X Social System	1	8.610E-7	.352	.564
Residual	12	2.446E-6		
Vs				
Sex	1	1.371E-7	.293	.597
Social system	1	3.190E-6	6.812	.021*
Sex X Social System	1	1.395E-7	.298	.594

Residual	14	4.683E-7		
Vv/Vl				
Sex	1	.000	.597	.454
Social system	1	.000	.701	.418
Sex X Social System	1	.000	.616	.447
Residual	13	.000		

*Significant differences within a testing treatment.

Chapter 4

Table 4.1. Partner fidelity among pairing species of butterflyfish.

Family and Genus	Species	Duration of partner fidelity*	Location	Ref.
Chaetodontidae				
Chaetodon				
	C. baronessa	1.5 months	Lizard Isl., GBR, Australia	1
	C. baronessa	4 months	Heron Isl., GBR, Australia	2
	C. chrysurus	3 years	Sinai cst, Red Sea, Egypt	3
	C. fasciatus	6 years	Sinai cst, Red Sea, Egypt	3
	C. lunulatus	1.5 months	Lizard Isl., GBR, Australia	1
	C. lunulatus	3 months	Kuroshima Isl., Japan	4
	C. lunulatus	6 months	Kuroshima Isl., Japan	5
	C. lunulatus	4 months	Heron Isl., GBR, Australia	2
	C. lunulatus	7 years	Heron Isl.	8
	C. multicinctus	> 7 months	Kona cst, Hawaiian Isls., USA	7
	C. multicinctus	> 4 years	Hawaiian Isl., USA	6
	C. unimaculatus	1 year	Eniwetok At., GBR, Australia	2
	C. ornatissimus	1 year	Kona cst, Hawaiian Isls., USA	7
	C. quadrimaculatus	1 year	Kona cst, Hawaiian Isls., USA	7
	C. vagabundus	1.5 months	Lizard Isl., GBR, Australia	1
Heniochus				

	H. intermedius	3 years	Sinai coast, Red Sea	3

*In each case, the duration of partner fidelity equals the duration of the study, and therefore should be considered a minimum value. *References:* [1]Nowicki, thesis chapter 1, [2]Reese, 1973, [3]Fricke, 1986, [4]Yabuta, 2000, [5]Yabuta, 1997; [6] Tricas, 1986, [7]Driscoll and Driscoll, 1988; [8] Reese, 1991.

Table 4.2. Standardized canonical coefficients (SCC) between canonical discriminant function (CDF_1) and response variables of *C. lunulatus* and *C. baronessa* to relationship phase (enduring vs. new partner) and day (day 1-5 = enduring partner; day ≥ 6 = new partner).

Response variable	Phase	Day
	CDF_1	CDF_1
	SCC	SCC
C. lunulatus		
Coordinated swimming	-0.37	-0.43
Within-pair agonism	0.61	0.61
Agonism per competitor rate	0.21	0.12
Feeding bite rate	-0.28	-0.29
Variance explained (%)	100	100
C. baronessa		
Coordinated swimming	0.56	0.60
Within-pair agonism	0.90	0.84
Agonism per competitor rate	0.19	0.11
Feeding bite rate	0.22	0.1
Variance explained (%)	100	100

Table 4.3. Means of standardized canonical scores of the first canonical discriminant function (CDF_1) for *C. lunulatus* and *C. baronessa* in response to relationship phase (with enduring partner vs. with new partner) and days (day 1-5 = with enduring partner; day ≥ 6 = with new partner).

	C. lunulatus	*C. baronessa*
	Mean	Mean
Phase		
Enduring	-0.63	-0.67
New	0.45	0.41
Day		

1	-0.66	-0.57
2	-0.62	-0.51
3	-0.67	-0.56
4	-0.53	-0.75
5	-0.61	-0.88
6	2.01	2.51
7	1.09	1.23
8	0.56	0.22
9	0.46	0.72
10	-0.01	-0.37
11	-0.55	-0.12
12	-0.48	-0.44
13	--	-0.23
	--	-0.61

Appendix B

Additional publications during my candidature:

1. Pratchett, M., **J. P. Nowicki**, A. Dewan, S. Walker, K. M. Chong-Seng, D. A. Feary, A. S. Hoey, C. J. Fulton, M. L. Berumen (2014). Butterflyfishes as a model group for reef fish ecology: Important and emerging research topics. In M.S. Pratchett; M. L. Berumen; B. G. Kapoor (Eds.), *Biology of Butterflyfishes*. CRC Press.

2. Coker, D., **J. P. Nowicki**, M. Pratchett (2015). Body condition of the coral-dwelling fish *Dascyllus aruanus* (Linnaeus 1758) following host colony bleaching. *Environmental Biology of Fishes.* 1-5.

3. Pratchett, M.S., S. Blowes, D. Coker, E. Kurbacki, **J. P. Nowicki**, A. Hoey (2015). Indirect benefits of high coral cover for non-corallivorous butterflyfish. *Coral Reefs.* 34.2: 665-672.