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Causes and possible consequences of hybridisation in angelfishes at Christmas Island

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Causes and possible consequences of hybridisation in angelfishes at Christmas Island

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… to my Family

Abstract

The angelfishes (family Pomacanthidae) have the greatest proportion (~30%) of hybridising species, compared to other families of reef fishes, with 26 species implicated in hybridisation. However, very little is known about hybridisation in angelfish, especially in terms of fitness of the hybrids and possible ecological consequences. Hybrids between three species (*C. flavissima, C. eibli and C. vroliki*) in the genus *Centropyge* have previously been reported from Christmas island, where these have been observed in heterospecific harems and interbreeding. This provides the unique opportunity to examine the breakdown in assortative mating in marine fishes. The broad aim of this study was to determine causes and consequences of hybridisation in angelfish at Christmas Island (Western Australia). To achieve this, the abundances, habitat and fitness of the three angelfish parent species and their hybrids have been investigated. Based on 14 years of surveys, *C. flavissima* was abundant (4.53 per 250 m^2 +/- 0.66), whereas *C. eibli*, *C. vrolikii*, and all hybrid combinations were consistently low in abundance or rare (average abundance < 0.3 per 250 m2 +/- 0.03). Parent species and their hybrids displayed high niche overlap, with all being more abundant at 20 m depth compared to 5 m, and showing similar diets that comprised a mix of green, red, and brown algae. Thus, rarity of parent species and niche overlap would help to promote hybridisation in angelfishes at Christmas Island.

The relative fitness of angelfish hybrids against the parent species was evaluated by comparing key life history traits. Both the von Bertalanffy growth coefficient (k) and the asymptotic length (L$_\infty$) of the hybrid *C. flavissima* × *C. eibli* did not differ from the parent species *C. flavissima*. In comparison, *C. eibli* tended to grow at a slightly faster rate but reached a smaller size than the hybrid and *C. flavissima*, potentially increasing its reproductive success. Both females and males of the hybrid *C. flavissima* × *C. eibli* presented similarly developed gonads and showed all stages of oocytes and spermatocytes development to the parent species. The presence of post-ovulatory follicles indicated possible spawning activity (and fertility) of the hybrid. The level of fitness of the hybrids at Christmas Island was similar to their parent species, and could explain their persistence at this location.

Angelfish hybrids formed harems with all parent species and the species composition of the harems reflected the underlying patterns of abundance for the parent species. The total number of fish and number of males did not differ between different types of harems (mixed and pure). Harems comprised an average of ~4 fishes, generally with one male, but some harems had 2 males, which were found in each type of harem. Almost one third of the dominant males in the mixed harems were hybrids, while ranks two to six were occupied by similar percentages of hybrids. The ability of the angelfish hybrids reaching similar ranks as both males and females of parent species and being in similar sized harems in mixed harems, combined with them displaying similar growth and size, indicates that their reproductive output would be similar to the parent species *C. eibli* and *C. vrolikii*. However, since *C. flavissima* was far more abundant and was the only species to form mono-specific harems, its reproductive output would be larger than the other two species and its hybrid.

This study provides empirical evidence that hybridisation in reef fishes conforms to terrestrial-based theories, and thus advances our understanding of the processes underlying hybridisation in coral-reef systems. Given the variable changes in environmental conditions occurring in the ocean, hybridisation and introgression could prove beneficial if it can provide new genotypes that increase the adaptive capacity of hybrids and/or parent species.

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LIST OF PUBLICATIONS INCLUDED AS PART OF THE THESIS

This thesis is presented as a series of manuscripts that are to be submitted for publication in international journals. I am the first author with a contribution of more than 50% to each publication. The thesis conforms to the "thesis with publication" style of Edith Cowan University.

I. Federico Vitelli, Jean-Paul Hobbs. *In prep.* **Review of natural hybridisation in angelfishes of the world.**

Journal targeted: ███████████████████

II. Federico Vitelli, Glenn A. Hyndes, Benjamin J. Saunders, David Blake, Stephen J. Newman and Jean-Paul A. Hobbs. **Do ecological traits of low abundance and niche overlap promote hybridisation among coral-reef angelfishes?**

Submitted to ███████

III. Federico Vitelli, Glenn A. Hyndes, Corey Wakefield, Stephen J. Newman and Jean-Paul A. Hobbs. **Hybridising angelfishes (genus *Centropyge*): comparative life history and fitness of hybrids and their parent species**

Submitted to ███████

IV. Federico Vitelli, Glenn A. Hyndes, Stephen J. Newman and Jean-Paul A. Hobbs. *In prep.* **Social structure of hybridising angelfishes at Christmas Island.**

Journal targeted: ██████

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Chapter 1. General introduction

1.1 Hybridisation

Hybridisation occurs through the interbreeding between two genetically distinct species (or populations), that produces viable offspring (Harrison 1993). Barriers often prevent reproduction between species through geographic isolation, mating behaviours, and genetic incompatibility between different species (Mayr 1942; Palumbi 1994). For example, different species might be geographically isolated and not able to become in contact and hybridise (Bowen et al. 2013), or the barrier of assortative mating can prevent the interbreeding (Jiang et al. 2013). Even when these barriers are overcome, hybrids might be sterile due to meiotic difference of parental chromosomes (allopolyploidy) (Seehausen 2004).

Hybridisation can occur when those barriers that are critical to speciation are overcome, and can potentially increase and decrease species diversity (Seehausen 2004, 2006). Regions where hybridisation occurs are referred to as hybrid zones, where genetically distinct populations, interbreed and produce hybrids (Barton and Hewitt 1989). Hybrid zones are generally small and are often located along the geographic borders between the two populations or species (Chen et al. 1997; Morgan et al. 2009). Biogeographic borders, where different regional communities come into secondary contact, often result in hybridisation between many species-pairs and are referred to as a suture zones (Remington 1968; Hewitt 2000). Natural or anthropogenic causes can interrupt geographic isolation and closely- related species might become in secondary contact and hybridise (Rhymer and Simberloff 1996). For example, the rising of ocean levels might have put in secondary contact sister species that diverged during the Pleistocene, enhancing the chances of hybridisation (Hobbs et al. 2009). Human activities can also directly or indirectly modify the environment, and alter species interactions (Grabenstain and Taylor 2017). For example, habitat homogenization, such as deforestation, can bring formerly isolated species in contact by reducing natural barriers, or human caused interferences (visual, acoustic, and/or chemical) could alter the ability of a species to recognise conspecific partners (Grabenstain and Taylor 2017).

Hybridisation has been documented for at least 10% of animal and 25% of plant species, but at the population level, hybrids represent 0.1% of individuals in a typical population (Mallet 2007). Hybrid individuals exhibit characteristics from each parent, and although some hybrids are unfit, other hybrids are able survive to reproduction and can occupy ecological niches that are different from the parent species (Mallet 2007). In general, the fitness of the hybrids is measured as the ability to reproduce and pass their genes to the next generation (Mills and Beatty 1979). Thus, a hybrid is defined unfit when sterile (e.g. the mule), or when it shows reduced fertility compared to its parent species (e.g. hybrids of annual sunflower) (Burke and Arnold 2000; Gardner et al. 2001). Conversely, those hybrids that survive and reproduce can play a vital role in the evolution of plants and animals (Barton 2001; Wissemann 2005), and can have both positive and negative outcomes (Richards and Hobbs 2015). For example, it could result in a 'macro-mutation' that allows the hybrid to colonize unoccupied ecological niches and increase their fitness (Barton 2001). On the other hand, extensive introgressive hybridisation could result in the extinction of one of the parental species or the fusion of the two species resulting in the loss of both parental species along with their discrete morphology, behaviour, and ecology (Willis et al. 2006).

1.2 Hybridisation in fishes

Hybridisation is observed in fishes more commonly than in other vertebrate groups, especially in freshwater fishes (Scribner et al. 2001). Much of the focus has been in freshwater fishes, but more recently several studies have indicated that hybridisation is common in coral-reef fishes (Hobbs and Allen 2014; DiBattista et al. 2016a; Montanari et al. 2016). From these studies, several factors have been proposed as contributing to the high incidence of hybridisation among closely related fish taxa, including: weak behavioural isolating mechanisms, unequal abundance of the two parental species, competition for limited spawning habitat, and shared ecological niches due to secondary contact between recently evolved forms (Scribner et al. 2001; Montanari et al. 2016). Rarity of one or both parent species may promote hybridisation, as an individual of a rare species, struggling to find a conspecific partner, can interbreed with an individual from a closely related and more abundant species (Hubbs 1955). Similarly, the sharing of the same resources such as habitat and food has been shown to promote hybridisation in other freshwater fish families (Scribner et al. 2001) and marine fish families such as

Chatodonthidae (Hobbs et al. 2013), and groupers from the genus *Cephalopholis* (Payet et al. 2016). The ecological niche overlap may increase the chances of encounters between individuals from different species and facilitate the formation of mixed species groups and/or mating pairs and subsequently hybridisation. In addition, Frisch and van Herwerden (2006) showed that hybridisation between two species of coral trouts may be promoted by the sharing of the same spawning sites. These factors may also act simultaneously, further increasing the chances of hybridisation (Hobbs et al. 2014; Montanari et al. 2016).

Coral-reef fishes form the most diverse vertebrate communities on earth and recent studies have revealed that hybridisation is common in this group (Richards and Hobbs 2015). Since the end of the nineteenth century, when the first probable natural marine fish hybrid was reported (Holt 1883), at least 111 new hybrids have been described, and most of them (~70%) come from tropical waters (Montanari et al. 2016). In their recent review on marine fish hybrids, Montanari et al. (2016) showed that 27 families were involved in hybridisation, and, most importantly, almost 40% of the reported hybrids were from the 2 families of *Chaetodontidae* (27 hybrids) and *Pomacanthidae* (16 hybrids). Those two families display a high incidence of hybridisation, and in most cases, the factors mentioned above were involved in promoting the process, especially the rarity of one or both parent species (Montanari et al. 2016).

Hobbs and Allen (2014) showed that hybridisation is common in coral-reef fishes at the Indo-Pacific biogeographic border, with Christmas and Cocos (Keeling) Islands being reported as a global hotspot for hybridisation (Hobbs et al. 2009). This biogeographic border is located in the eastern Indian Ocean and is considered a marine suture zone because it represents an area where many Indian Ocean and Pacific Ocean species come into secondary contact and hybridise. At Christmas and Cocos Islands, hybridisation is mainly occurring between Indian Ocean and Pacific Ocean sister species that diverged in allopatry when Pleistocene low sea-levels caused land bridges throughout Indonesia (Hobbs et al. 2009). So far, 15 hybrids involving 27 species across eight families have been reported from Christmas Island and Cocos Keeling Island (Hobbs and Allen 2014). Most of the species involved in hybridisation were from the *Chaetodontidae* family (n=8) followed by *Acanthuridae* (n=6) and *Pomacanthidae* (n=3).

1.3 Angelfishes and hybridisation

Angelfishes show a wide range of ecological traits, such as body size, colour, reproductive system, and diet (Allen et al. 1998), and many studies have examined these different aspects at the species level (Perez-Espana and Abitia-Cardenas 1996; Bellwood et al. 2004; Arellano-martínez et al. 2007; Callan et al. 2012). Angelfishes comprises about 88 species divided into 8 genera. The biggest genus is *Centropyge* which includes 32 species, followed by *Chaetodontoplus* with 14 species (Kuiter et al. 2003). The most diagnostic character of all angelfishes is a pronounced spine protuding posteriorly from the lower corner of the preopercle. This is a unique characteristic of angelfishes visible in the juvenile to adult stages, that distinguish them from similar families such as butterflyfishes and surgeonfishes (Kuiter at al. 2003). Most angelfishes have a compressed rhomboid body and high variety of beautiful colorations, that make them a target for aquarium trades (Allen et al. 1998). Most species usually feed on algae and sponges and some species are planktivorous (Kuiter et al. 2003). Angelfishes occur in tropical and subtropical ecosystems and most of the species (~89%) inhabit the Indo-Pacific (Allen et al. 1998). Most of the angelfish species are haremic, and others form mating pairs (Allen et al. 1998). Harems usually consist of a dominant male and two to six females. At dusk, the male starts chasing the females until he finds one that follows him, then they both swim up in the water column and at apex of their ascent they release their gametes. The male usually spawns with each member of the harem, and most of the species spawn throughout the year (Moyer et al. 1983; Allen et al. 1998).

The angelfishes (family Pomacanthidae) have the greatest proportion (~30%) of hybridising species compared to the other families of coral-reef fishes, with 26 species implicated in hybridisation (Yaakub et al. 2006). However, very little is known about hybridisation in angelfish, especially in terms of the possible causes and ecological consequences of hybridisation (Pyle and Randall 1994). The pygmy angelfishes (genus: *Centropyge*), which are well known for their bright colouration, comprise the greatest number of species (34) in the family (Allen et al. 1998). These angelfishes maintain a haremic mating system and exhibit protogynous sex change, with the largest female in a harem undergoing sex change after the disappearance of the dominant male (Sakai et al. 2003). Three species (*C. flavissima, C. eibli and C. vroliki*) have been observed in heterospecific harems and interbreeding at Christmas island, with hybrids being observed

in the region (Hobbs and Allen 2014), thus, they provide a unique opportunity to examine the possible causes and consequences of hybridisation in marine fishes.

1.4   Aims and structure

This project aims to determine causes and consequences of hybridisation in angelfishes using three hybridising angelfish species from the genus *Centropyge* at Christmas Island at the suture zone in between the Indian and Pacific Oceans. In order to achieve this aim, the project has been divided into four studies, with each forming a chapter. Each chapter has been developed and presented as a paper with its individual aim and research questions, and these have either been, or being prepared to be, submitted to journals. Chapter 2 is a review of what is known so far about hybridisation in angelfishes. Chapters 3, 4 and 5 are based on field studies at Christmas island to examine aspects of hybridisation. In Chapter 3, the abundances, diet, macro- and micro-habitat use of three parent species and their hybrids were investigated to understand the causes of hybridisation. In Chapter 4, fitness of hybrids and parent species was evaluated in terms of growth and reproduction, to determine whether hybrids exhibit similar growth, maturity, and reproductive characteristics as their parent species. Chapter 5 examined the social status of the hybrids inside harems by determining the rank and sex of the hybrids compared to their parent species in harems to establish whether or not the hybrids exhibited a similar capacity to reproduce as their parent species. Finally, Chapter 6 synthesises and discusses the findings from the previous chapters and other studies at different locations of the world aiming to provide a clearer view of causes and consequences of hybridisation in angelfishes. This approach leads to a discussion on the ecological and management implications of such findings in the perspective of a changing environment.

Chapter 2. Review of natural hybridisation in angelfishes of the world

2.1 Introduction

Hybridisation is defined as the interbreeding between two genetically distinct populations or species, that produces viable offspring (Harrison 1993). Hybridisation is an important process that has been documented in animal (10%) and plant (25%) species (Mallet 2007). This process can have important evolutionary consequences such as the increase and decrease in biodiversity (Seehausen 2004, 2006), and has been well studied in terrestrial and freshwater environments, but has traditionally been considered rare in marine systems (Arnold 1997). Studies from terrestrial and freshwater environments have shown that hybridisation is more likely to happen between species that share the same niche; this will, in fact, increase the chances of encounters between different species (Arnold 1997; Scribner 2001). In addition, rarity of one or both parent species has been considered an important factor involved in hybridisation (Arnold 1997; Scribner 2001; Mallet 2007).

Depending on the fitness of hybrids, different evolutionary scenarios can take place. Some hybrids are unfit, and are considered "dead-ends", while others are able survive and reproduce, and might have the potential to occupy different ecological niches than their parent species (Mallet 2007). Thus, hybridisation can play a vital role in evolution of plants and animals (Barton 2001; Wissemann 2005), and can have both positive and negative outcomes. For example, through genetic recombination, hybrids could be able to exploit unoccupied niches or be able to tolerate challenging environmental conditions (Barton 2001). On the other hand, hybridisation can lead to extensive introgression that results in two species blending into one (reverse speciation) (Seehausen 2004), thus resulting in a loss of biodiversity.

Hybrid zones are areas where genetically distinct populations interbreed and produce hybrids, and these areas provide valuable research opportunities to gain unique insights into evolutionary processes (Barton and Hewitt 1989). Hybrid zones are generally small

and often represent narrow areas of overlap in the distribution of the two parent species (Harrison 1993; Hobbs et al. 2009; DiBattista et al. 2015). Biogeographic borders where regional biotas come into secondary contact often result in hybridisation between many species-pairs and are referred to as a suture zones (Remington 1968; Hewitt 2000).

Hybridisation is most commonly reported in fishes (Scribner et al. 2001), with freshwater fishes being reported to hybridise more than marine fishes. However, this may reflect sampling bias and the longer history of research on freshwater fishes. Based on those studies, several biological, behavioural, ecological and genetic factors have been proposed as contributing to the high incidence of hybridisation in freshwater fishes, including: close-relatedness between parent species, weak behavioural isolating mechanisms, unequal abundance of the two parental species, competition for limited spawning habitat, shared ecological niches and secondary contact between recently diverged forms (Hubbs 1955; Scribner et al. 2001).

Although research on hybridisation in marine fishes has lagged that on freshwater fishes, there has been a rapid rise in the amount of research on this topic over the past two decades (Richards and Hobbs 2015; Montanari et al. 2016). This provides a timely opportunity to assess the causes and consequences of hybridisation in marine fishes. Among marine fishes, two groups account for approximately 40% of species reported to hybridise: butterflyfishes (family Chaetodontidae) and angelfishes (family Pomacanthidae) (Montanari et al. 2016). A recent review of hybridisation in butterflyfishes found rarity of parent species, shared ecological niches, breakdown in assortative mating, range edges, secondary contact and genetic relatedness were linked to hybridisation (Hobbs et al. 2013). Whether these findings are representative of marine fishes more generally requires testing on other groups where hybridisation is common.

The marine fish family with the second highest number of hybridising species is angelfishes (Montanari et al. 2016), which is the sister group to butterflyfishes (Bellwood et al. 2004). Angelfish are predominately found on coral reefs, and most species live at depths between 5 and 60 m (Allen et al. 1998). They show a wide range of body sizes (5 to 50 cm TL), colour, reproductive system, and diet (Allen et al. 1998). Hybridisation in angelfishes was first reviewed by Pyle and Randall (1994) who provided a detailed account of the evidence supporting hybridisation in 11 probable, and 5 possible, hybrid

crosses. Since then, there have been numerous reports of new angelfish hybrids, with Yaakub et al. (2006) and Montanari et al. (2016) finding reports of 26 species and 23 species, respectively, involved in hybridisation. There has also been a range of recent studies into the behavioural, ecological and genetic studies on hybridising angelfishes (Hobbs et al. 2009; Hobbs et al. 2010; DiBattista et al. 2012; Hobbs and Allen 2014; Gaither et al. 2014). Four angelfish phylogenies have also been published that provide insights into evolutionary relationships between hybridising species (Bellwood et al. 2004; Hodge et al. 2013; Gaither et al. 2014; Tariel et al. 2016). These recent studies provide the opportunity to investigate the causes and consequences of hybridisation in angelfishes and whether these patterns reflect those found in butterflyfishes (Hobbs et al. 2013), other marine fishes (Montanari et al. 2016), and freshwater fishes (Scribner et al. 2001).

The overall aim of this study is, through reviewing available literature and data, to examine the causes and consequences of hybridisation in marine angelfishes. The specific aims are to determine:

1. The geographic distribution of hybridisation in angelfishes and whether there are hybrid hotspots
2. If hybridisation is more common in angelfish species that have a narrow overlap in geographic ranges (i.e. parapatry) compared to species that have broadly overlapping ranges (i.e. sympatry).
3. If there is taxonomic bias to hybridisation in angelfishes
4. If rarity of one or both parent species promotes hybridisation due to a lack of conspecific partners.

2.2 Methods

We conducted a search of the literature using search engines/databases including: Web of science, Google scholar, Reef builders, Iucn redlist, and Fishbase. The following search terms were used: angelfish, hybrid, hybrids, hybridisation, angelfish hybrid, fish hybrid, angelfish hybridisation, hybridisation in angelfish, *Centropyge* hybrid, *Paracentropyge* hybrid, *Genicanthus* hybrid, *Apolemichthys* hybrid, *Chaetodontoplus* hybrid, *Pygoplites* hybrid, *Pomacanthus* hybrid, *Holacanthus* hybrid. We also examined all known guidebooks for angelfishes (Allen et al. 1998; Kuiter et al. 2003; Michael 2004;

Endoh 2007). Angelfishes have bright and distinctive colour patterns and are among the most popular fishes in the marine ornamental trade/aquarium industry due to their bright and distinctive colouration. Hybrid fishes are usually first detected by their intermediate colouration and the keen eye of collectors and aquarists has been credited to providing scientists with the first specimens of new angelfish hybrids (Pyle and Randall 1994). For this reason, we have also included reports of angelfish hybrids from collectors/aquarists where descriptions and images are provided. For every putative angelfish reported by scientists or aquarists we outline the supporting evidence.

To determine if hybridisation in angelfishes is linked to rarity of parent species, we used reports of abundance from reputable sources (Allen et al. 1998; Michael 2004; Kuiter et al. 2003; Endoh 2007; www.iucnredlist.org). We supplemented these data with those from field surveys conducted between 2002 and 2015 (unpublished data). Field surveys were conducted between 5 and 25 m at 2 locations within the Indo-Pacific (Cocos Keeling Islands and Christmas Island). For the field surveys, a species was considered rare if its average density was less than 3 fish per 1 hr dive or approximately 1500 m^2. For locations that were not surveyed, rarity of a species was based on reports of abundance from reputable sources (Allen et al. 1998; Kuiter et al. 2003; Michael 2004; Endoh 2007; www.iucnredlist.org).

To determine if hybridisation occurs between angelfishes that have a narrow area of overlap, we examined geographic distributions using guidebook (Allen et al. 1998; Kuiter et al. 2003; Michael 2004; Endoh 2007) and IUCN website (www.iucnredlist.org). We categorised parent species as having either parapatric distributions with a narrow area of overlap (<20% of their ranges) or broadly sympatric distributions (>20% overlap in their ranges). Due to fine scale habitat data not being available for all species, we used depth range of the parent species from Allen (1998) as a broad measure to determine the overlap of habitat, recognizing that this is a broad measure of habitat overlap.

2.3 Number of hybrids, genera and species involved

The last review on hybridisation of angelfish was done in 1994 by Pyle and Randall. They reported evidence for 11 probable pomacanthid hybrids from three different genera (*Apolemichthys*, *Centropyge* and *Pomacanthus*), and described five more possible

hybrids in *Centropyge*, *Chaetodontoplus* and *Pomacanthus*. In this review, we provide evidence for 31 hybrids involving 36 species in 7 out of 8 genera of Pomacantidae. Considering that the family Pomacanthidae includes approximately 88 species (Kuiter et al. 2003), 41% of angelfish species are involved in hybridisation. Hobbs et al. (2013) showed that 38.6% of Chaetodonthidae species are known to hybridise, putting butterflyfishes on top or the list for both absolute number of hybridising species (44) and percentage. With this review, we put angelfishes on top of the list for percentage of species involved in hybridisation. The two percentages are very close, and are much higher than other families of marine fish (Yakuub et al. 2006), but also plants (25%) and animals (10%) in general. In addition, the number of angelfish species involved in hybridisation could be higher because of the difficulty to identify hybrids and to explore deep habitats such as the mesophotic coral ecosystems (Hinderstein et al. 2010).

Coral-reef fish hybrids of each family are usually concentrated within just few genera (Yakuub et al. 2006), for example, most of the hybrids within the family of Chaetodontidae occur from interbreeding species within the genus *Cheatodon* (Hobbs et al. 2013). In contrast, angelfish hybrids have been reported from 7 out of 8 genera (Table 2.1). In particular, we report 13 hybrids involving 12 species of the genus *Centropyge*, 3 hybrids involving 5 species of *Genicanthus*, one hybrid from two species of *Paracentropyge*, three hybrids from 4 species of *Apolemichthys*, two hybrids from 4 species of *Holacanthus*, 9 hybrids from 9 species of *Pomacanthidae*, and 2 more possible hybrids involving three species in the genus *Chaetodontoplus*. All these hybrids occur in their natural habitats. In addition, 6 additional hybrids have been created in captivity (see Table 2.1). Most of the angelfish hybrids occur within the genera *Centropyge* and *Pomacanthus*, which could be because of the high number of species within the 2 genera compared to the other genera, and also because of the popularity of those fish for aquariums and underwater photographers.

Table 2.1 Parental species for reported hybrids. The table shows (from left to right): species names, species range (1=sympatric, 2= allopartic, 3=narrow contact zone), the location from where the hybrids were reported, whether the species were at their distribution edge (0=no parent species, 1=one parent species, 2=both parent species), rarity of parent species (0=no parent species, 1=one parent species, 2=both parent species), whether the depth range of the two parent species overlapped by 50% or more, and the supporting references (1=Allen et al. 1998; 2=Hobbs et al. 2009; 3= Pyle and Randall 1994; 4= DiBattista et al. 2012; 5=www.iucnredlist.org; 6=Michael 2004; 7= Endoh 2007; 8=Kuiter et al. 2007; 9=Moyer 1981; 10=www.reefbuilder.com; 11=Feddern 1968; 12=Feeley et al. 2009; 13=Reyes-Bonilla et al. 2012; 14=Tariel et al. 2016; 15= Sala et al. 1999; 16=Sanchez-Alcantara et al. 2006; 17=Alva-Campbell et al. 2010; 18=hodge et al. 2013; 19=Conde 1990; 20=Bock 1996; 21= https://reefs.com/magazine/evolution-biogeography-chaetodontoplus; 22=Krupp and Debelius 1990; 23=Kemp 2000).

Hybrid (sp. 1 x sp. 2)	Range	Location	Parent species at range edge	Rare at hybrid zone	Depth overlap by 50%	References
C. eibli × C. flavissima	3	Christmas and Cocos Island	2	1	yes	1,2,3,4
C. eibli x C. vroliki	3	Indonesia and Christmas Island	2	2	yes	1,2,3,4
C. flavissima x C. vroliki	1 or 3 depending on % overlap and vagrants	Christmas and Cocos Island, Guam, Micronesia, Marshall Islands, Vanuatu	2	1	yes	1,2,3,4
C. loriculus x C. shepardi	3	Guam and Micronesia	2	1	yes	1,5,6,7,8
C. loriculus x C. potteri	3	Hawaii	2	1	no	1,5,6,7,9
C. bispinosa x C. shepardi	3	Guam	1	1	yes	1,3,5,6,7,8,9
C. bispinosa x C. multicolor	2	Philippines, Marshal Island	1	2	no	5,10
C. ferrugata x C. multicolor	2	Philippines	1	1	no	5,10
C. fisheri x P. potteri	1	Hawaii	0	1	yes	5,10
C. ferrugata x C. loriculus	2	Philippines	1	1	yes	5,10
C. ferrugata x C. loriculus x C. bispinosus	2	Philippines	1	1	yes	5,10
C. heraldi x C. bicolor	3	Philippines	1	1	yes	1,5,6,7,8,9
C. bispinosa x C. heraldi	3	Philippines	1	1	yes	1,3,5,6,7,8,9
Genicanthus takeuchi x G. semifasciata	3	Ogasawara Island	2	1	yes	1,5,6,7,10
Genicanthus bellus x G. lamarck	1	Indonesia	2	0	no	1,5,6,7,10
Genicanthus melanospilos x G. lamarck	1	Japan	0	0	yes	1,5,6,7,11
Paracentropyge multifasciatus x P. venustus	1	Philippines	1	1	yes	1,5,6,7,11,22
Apolemichthys trimaculatus x A. xanthurus	3	Seychelles and Maldives	2	1	no	1,3,5,6,7,8,
A. trimaculatus x A. kingi	3	South Africa	2	1	yes	5,6,7,8
A. trimaculatus x A. xanthopunctatus	3	Kiribati	2	0	yes	5,7
Holacanthus bermudensis x H. ciliaris	1	Bermuda, Florida Keys, South Carolina, Bahamas, St Eustatius	1 or 2 depending on locations	1 or 0 depending on locations	yes	1,3,5,6,7,8,11,12,13,14
H. passer x H. clarionensis	3	Baja California, Mexico	2	1	yes	1,5,6,7,8,14,15,16,17
Pomacanthus maculosus x P. asfur	3	Kenya	2	1	yes	1,5,6,7,8,18
P. sextriatus x P. xanthometopon	3	Queensland, Australia and Bali	1	0	yes	1,3,5,6,7,8,18
P. navarchus x P. xanthometopon	1	Indonesia, Philippine, Queensland Australia	2	0	yes	1,3,5,6,7,8,19
P. chrysurus x P. maculosus	3	Kenya	0	2	yes	1,3,5,6,7,8,10,18,19
P. maculosus x P semicirculatus	3	Kenya and Oman	2	2	yes	1,3,5,6,7,8,10,18,19,20,23
P. chrysurus x P semicirculatus	3	East Africa	2	2	yes	1,3,5,6,7,8,10,18
P. imperator x P. chrysurus	1	East Africa	2	1	no	1,3,5,6,7,8,10,18
P. imperator x P. semicirculatus	1	East Africa	2	1	yes	1,3,5,6,7,8,10,18
P. rhomboides x P. semicirculatus	1	South Africa	2	1	yes	1,3,5,6,7,8,10,18
Chaetodontoplus melanosoma x C. septentrionalis	3	Taiwan	2	1	yes	1,3,5,6,7,8,21
C. caeruleopunctatus x C. septentrionalis	1	Japan	2	1	yes	1,3,5,6,7,8,21

2.4 Distribution of angelfish hybrids - Hot spots and suture zones

The distribution of marine fish hybrids is usually concentrated in particular locations called suture zones (Gardner 1997). However, angelfish hybrids have been observed and reported from 24 locations across the three oceans of the world. The highest number of hybrids was observed in the Philippines (n=8) and East Africa (mainly Kenya, n=6), followed by Indonesia (n=4), Christmas Island, Guam, and Japan (n=3), Cocos Island (WA), Marshall Islands (US), South Africa, Micronesia and Queensland (n=2), and single hybrids where they were also reported from Seychelles, Taiwan, Maldives, Kiribati, Bermuda, Florida Keys, Bahamas, Saint Eustatius (Caribbean), Mexico, and Oman (Fig. 2.1). Several hot spots of hybridisation have been reported, such as the Philippines, Taiwan, Great Barrier Reef, and Indonesia (Allen et al. 1998). The wide spread distribution of angelfish species is reflected by their hybrid distribution, with the highest number of hybrids concentrated mainly where numbers of angelfish species are high (Allen et al. 1998).

Most cases of hybridisation were recorded along previously proposed biogeographic borders (Fig 2.1). This is also common in terrestrial cases of hybridisation (Hewitt 1988). The reasons why different species mainly interbreed at biogeographic borders are because: (1) recently diverged (allopatric) species in these areas can come into secondary contact, increasing the chances of interbreeding (Scribner et al. 2001; Hodge et al. 2013); and (2) biogeographic borders usually represent the distribution edge of many species, which is where species abundances usually decrease (Brown 1984). These factors act simultaneously, increasing the chances of hybridisations. The low abundances of species in these areas can force an individual to interbreed with a once separated but closely-related species (allopatric) if it is not able to find a conspecific partner (Hobbs et al. 2013). In angelfishes, only 4 cases of hybridisation are likely to reflect secondary contact of allopatric diverged species, while in 30 (90%) cases, at least one of the two parent species was at its range edge. In 11 of 35 cases of hybridisation, the parent species were sympatric, however, hybridisation was still recorded in locations located at the range edge of one or both species. In addition, 19 cases were recorded in locations of narrow overlap between the two parent species, meaning that both were at their range edges. Many cases of hybridisation were also recorded at isolated islands or atolls, which are usually

characterised by vagrant and endemic species with low abundances, further increasing the chances of hybridisation (Rhymer and Simberloff 1996; Hobbs et al. 2009). Indeed, these peripheral locations presents peculiar conditions that, in different ways, favour hybridisation.
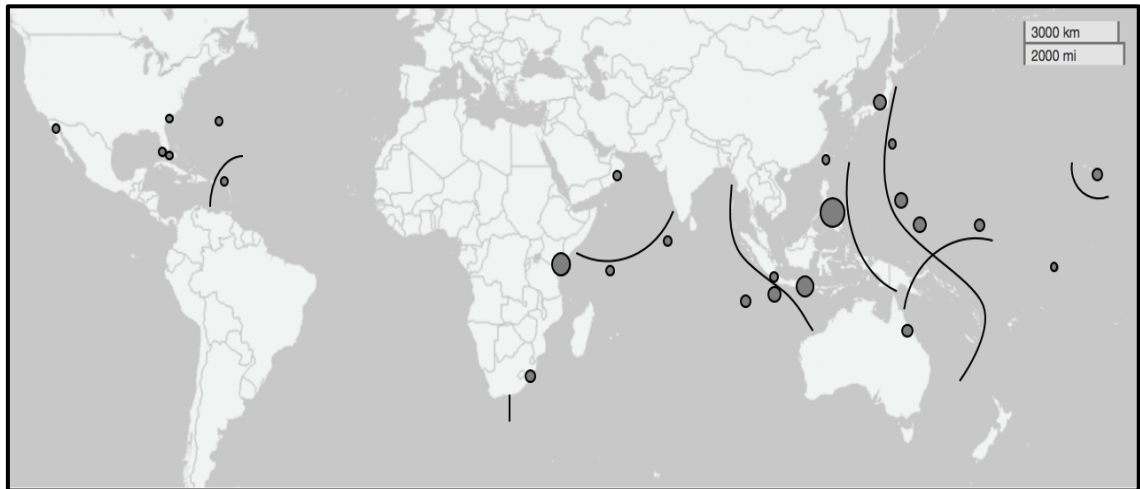
Figure 2.1 The geographic distribution of reported locations of angelfish hybrids. The size of the circle is indicative of the number of species pairs that have produced hybrids at a reported location, with the largest circles symbolising eight cases, and the smallest one case. The black lines represent recognised biogeographic borders (Blum 1989; McMillan and Palumbi 1995; Bellwood and Wainwright 2002; Hsu et al. 2007).

2.5 Abundance disparities and niche overlap

In both terrestrial and marine environments, rarity of one or both parent species has promoted hybridisation (Mallet 2007; Hobbs and Allen 2014). An individual of a rare species might interbreed with an individual from a closely related species if it is not able to find a conspecific partner. Based on the published data, and data from field surveys in the Indo-Pacific, at least one of the two parent species were rare in 31 of 33 cases of hybridisation. In two cases, hybridisation occurred between two common parent species (Japan and central Indonesia). In these locations, hybridisation might be caused by other factors, such as habitat and/or diet overlap. These factors have been showed to promote hybridisation in freshwater and marine environments (Scribner et al. 2001; Montanari et al. 2012, 2016; Hobbs and Allen 2014; Richards and Hobbs 2015; Payet et al. 2016).

Angelfishes have different mating strategies, most species maintain a haremic mating system and exhibit protogynous sex change (Sakai et al. 2003), and other species form mating pairs (Allen et al. 1998). Sharing the same resources such as food and habitat can increase the chances of encounters between individuals of different species. This will facilitate the formation of heterospecific harems or pairs, and contribute to the breakdown in assortative mating and subsequent hybridisation. In 27 of 33 cases, the depth distribution of the two parent species overlapped by 50% or more. This overlap is likely to facilitate encounters between different species, especially for haremic and pair forming species such as angelfishes. Diet overlap is also an important factor promoting hybridisation (e.g. Montanari et al. 2012), however, this was not included in this review due to the limited availability of dietary data at the appropriate taxonomic resolution.

2.6 Discussion

Firstly, it is important to highlight the assumptions of this study. Here, we have assumed that every reported hybrid is actually a hybrid. While we have provided a range of evidence supporting hybridisation in each case, hybridisation is best confirmed using molecular approaches. Genetic studies of hybridisation are increasing. These studies genetically confirmed that individuals identified as hybrids by intermediate colouration are hybrids. For example, Christmas Island has the greatest number of reported hybrid marine fishes and all genetic studies have so far confirmed hybridisation (Hobbs and Allen 2014). Hybrids are underestimated (Mallet 2007) and can be "accidently" discovered during genetic studies of marine fishes (e.g. Kuriiwa et al. 2007). Thus, it is likely that most of the reported hybrids will be genetically confirmed in time, and that other unreported hybrids will be detected in genetic studies. There is no reason to suspect that there would be any biases (e.g. taxonomic) in any falsely reported hybrids. Thus, our conclusions are unlikely to change with the number of confirmed hybrids.

We found 33 cases of reported angelfish hybrids. Our analyses of biogeographic, phylogenetic, and ecological data revealed that rarity of one or both parent species and a strong depth range overlap of the two parent species are factors linked to hybridisation in angelfish. This usually happens between closely related species and at locations placed at their distribution edges. If one or both parent species are rare, an individual might struggle to find a conspecific partner, forcing it to mate with an individual of a different species

(Hubbs 1955). Depth range and habitat overlap has been shown to promote hybridisation in marine fish families such as butterflyfishes (Montanari et al. 2012; Hobbs et al. 2013), groupers (Payet et al. 2014) and angelfishes (Vitelli et al. 2018, submitted). Inhabiting the same macrohabitat, and having similar depth range distribution, will increase the chances of encounters between individuals of different species. This could promote the formation of heterospecific pairs or harems, and potentially lead to hybridisation (Frisch and van Herwerden 2006; Camp et al. 2016).

In most cases, hybridisation occurred at locations on the edge of the distribution range of one or both parent species, and usually at least one of the parent species was rare. Each of these factors can act singularly or cumulatively. The edge of geographic ranges usually represents a biogeographic border and species at these locations often have lower abundances (Brown 1984). This, combined with the species having similar habitat and diet, may put individuals of different species in contact with each other (Hobbs and Salmond 2008), and thus facilitate hybridisation. Indeed, these factors are interrelated, and may act simultaneously to favour the breakdown of the assortative mating and subsequent production of hybrids. The fact that most of the cases of hybridisation have been reported from locations that are close to biogeographic borders can be related to the particular conditions listed above that are associated with these sites, rather than the location themselves. In some cases, hybridisation occurs between two sympatric species, and both may be common. However, these species usually hybridise at the edge of their ranges where their abundances might be low. This condition may increase the possibility of encounters between closely-related species with similar ecological and behavioral traits. Once mixed pair or harems are formed, and the barrier of assortative mating are broken, the two parent species are likely to interbreed and produce hybrids.

If the hybrids are viable and fit enough to reproduce and inhabit the same territories of the parent species, they can evolve as a separate species, increasing the biodiversity (e.g. *Centropyge shepardi*) or result in the fusion of the two parent species with loss of biodiversity (reverse speciation). Indeed, due to climate changes and human activities, oceans environment is changing. The increasing acidity and temperature are causing loss of resources such as habitat and food for coral-reef fishes (Hughes et al. 2017), and challenging the physiology of the fishes (Hoey et al. 2016). If hybrids are viable, they have the potential to breed back with their parent species, creating a high gene flow. This

could give them the opportunity to evolve and be able to colonize new niches or survive in spite of challenging environmental conditions. The increasing alteration of the marine environment is likely to enhance the factors promoting hybridisation such as lack of conspecific partners, and resources overlap. For example, coral bleaching can lead to loss of food resources for butterflyfish and the decreasing of their abundances at the affected locations (Wilson et al. 2006). Under these conditions, hybridisation in the marine environment is likely to increase in the future, and thus, it is important to fully understand this process and its potential ecological consequences.

2.7 Future directions

The high number of angelfish hybrids, their haremic and pair mating systems, and their wide distribution around the world, make this family ideal to study the causes and consequences of hybridisation in coral-reef fishes. Many studies on hybridisation have been done in the terrestrial environment, thus, the starting point to understand hybridisation in marine environment will be to test whether terrestrial theories apply to marine ecosystems. To start with, a comprehensive ecological study would be ideal to investigate whether the factors promoting hybridisation in terrestrial and freshwater environments, such as niche overlap and abundance disparities, are also facilitating hybridisation in marine ecosystems.

The next step, after understanding the causes of hybridisation, would be to investigate the possible evolutionary consequences of this process. For example, are the hybrids viable? What's the fitness of the hybrids compared to their parent species? To achieve that, a biological study of the hybrids and their parent species is needed to evaluate and compare their fitness, especially in terms growth rates and reproductive output/success.

For haremic species, the bigger fish is usually the dominant male in the harem, which reproduces with multiple females (Moyer et al. 1983; Moyer and Zaiser 1984). Thus, males are able to mate multiple times during the day compared to a female that only spawn once (Moyer et al. 1983). The above studies can be combined with in vitro fertilisation experiments and genetic analysis to determine whether the hybrids are an evolutionary "dead-end" or whether they are viable and potentially play a vital role in the evolution process. To be reproductively successful, it is also important to be socially

accepted and overcome the barriers of assortative mating (Brock 1989). Mate choice experiments could be undertaken to understand mating preferences of the hybrids in combination with the collection and sexing of hybrids and parent species from natural mixed harems and mating pairs to better understand the social rank of the hybrids in their natural environment.

Finally, marine ecosystems are affected by climate change resulting in habitat loss and altered environmental conditions (Hughes et al. 2017). Hybrids could have the potential to inhabit new niches or adapt to survive challenging environmental conditions (e.g. increasing water temperature and pH). A comparison of the diet and habitat of the hybrids and their parent species, combined with a physiological study, could help to understand the capacity of adaptation of the hybrids and their potential to evolve as new species.

In conclusion, a high number of studies on marine hybrids have focused on genetics, trying to understand the taxonomy and to investigate whether or not the hybrid was a result of the interbreeding between different species (DiBattista et al. 2012, 2016b; Gaither et al. 2014). Indeed, genetic studies are important, especially to validate putative hybrids yet to be confirmed. However, it is important to implement this study with behavioural and ecological investigation to fully understand the causes and consequences of hybridisation in marine ecosystems, as highlighted in recent studies (e.g. Montanari et al. 2016).

Chapter 3. Do ecological traits of low abundance and niche overlap promote hybridisation among coral-reef angelfishes?

This chapter is not included in this version of the thesis.

Chapter 4. Hybridising angelfishes (genus *Centropyge*): comparative life history and fitness of hybrids and their parent species

4.1 Abstract

Hybridisation can influence evolutionary processes in positive and negative ways, depending on the fitness of the hybrids. However, we know very little about the consequences of hybridisation in marine ecosystems. Among marine fishes, angelfishes are well known for their high proportion (~30%) of hybridising species, and three species from the genus *Centropyge* (*C. flavissima*, *C. eibli* and *C. vrolikii*) have been reported to hybridise at Christmas Island (Indian Ocean). This study evaluated the relative fitness of angelfish hybrids against the parent species by comparing important life history traits. Both the growth coefficient (k) and the asymptotic length at an infinite age (L∞) of the hybrid *C. flavissima* × *C. eibli* were not different to one of the parent species *C. flavissima*. In contrast, the von Bertalanffy growth parameters k and L∞ of the hybrid were different to the parent species, *C. eibli*. *C. eibli* tended to grow at a slightly faster rate than the hybrid and *C. flavissima*, potentially increasing its reproductive success. However, *C. eibli* showed the lowest L∞ (95 vs 100 and 103 mm), and since fecundity is related to body size, lifetime reproductive output may potentially be less. Both females and males of the hybrid *C. flavissima* × *C. eibli* presented similarly developed gonads and showed all stages of oocytes and spermatocytes development to the parent species. The presence of post-ovulatory follicles indicated possible spawning activity (and fertility) of the hybrid. The level of fitness of the hybrids at Christmas Island was similar to their parent species, and could explain their persistence at this location. Given the variable changes in environmental conditions occurring in the ocean, hybridisation and introgression could prove beneficial if it can provide new genotypes that increase the adaptive capacity of hybrids and/or parent species.

4.2 Introduction

Hybridisation can influence the evolution of plants and animals in both positive and negative ways, and this is largely dependent on the fitness of the hybrids (Barton 2001;

Arnold and Martin 2010). Hybrids can show lower, equal or increased fitness compared to their parent species (Arnold and Hodges 1995). Lower fitness of the hybrids can be expressed as lower abundances, fertility or survival rates compared to parent species (Miller et al. 2004; Yaakub et al. 2007; Montanari et al. 2012), and ultimately hybrids can be an evolutionary dead end (e.g. sterile like the mule). Greater fitness can result in hybrids outperforming parent species in the same environment, or enable hybrids to exploit unoccupied niches or survive in challenging environmental conditions (Seehausen 2004; Karrenberg et al. 2006). Ultimately, increased fitness in hybrids can result in the generation of new species and adaptive radiations (Seehausen 2004).

The fitness of animals is defined as the ability to survive and reproduce (Mills and Beatty 1979). Animals that evolve greater fitness will live longer, grow faster, mature earlier and produce a higher number and larger eggs, and therefore produce more offspring over their lives (Brommer 2000; Mayr 2002). Hybrids of some terrestrial animal and plant species have shown to be able to grow faster and bigger than their parent species (Licht and Bogart 1989; Burke et al. 1997; Arnold and Martin 2010). Ultimately, if hybrids have greater fitness than the parent species, they will become the most numerically abundant and outcompete the parent species, and potentially the parent species may become extinct in locations dominated by hybrids (Rhymer and Simberloff 1996).

Hybridisation has traditionally been considered rare and unimportant in marine ecosystems (Arnold 1997). However, recent studies on hybridisation in marine ecosystems have shown it is common, particularly in coral-reef fishes, and especially in butterflyfishes (family: Chatodonthidae) and angelfishes (family: Pomacanthidae) (Pyle and Randall 1994; Hobbs et al. 2014; Hobbs and Allen 2014). However, few studies have tested the fitness of reef fish hybrids (Payet et al. 2016; Montanari et al. 2017), and therefore the evolutionary implications of hybridisation are largely unknown. Based on limited studies of growth, reproduction, and abundance, grouper and butterflyfish hybrids have exhibited a similar level of fitness to their parental species (Payet et al. 2016; Montanari et al. 2017).

Hybrids between three species of angelfishes from the genus *Centropyge* (*C. flavissima*, *C. eibli* and *C. vrolikii*) have been reported from Christmas Island in the Indian Ocean (Hobbs and Allen 2014). The three parent species and hybrids have overlapping niches

(habitat and diet: Vitelli et al. unpublished), yet there are large differences in abundances (Hobbs et al. 2010). This provides an ideal opportunity to test the fitness of the hybrids and understand possible evolutionary implications of hybridisation in coral-reef fishes. The aim of this study is to evaluate the relative fitness of angelfish hybrids against the parent species of the genus *Centropyge* from the Indo-Pacific suture zone at Christmas Island to determine whether angelfish hybrids exhibit different fitness to their parent species in terms of: (1) growth; (2) size at sexual maturity and sex change; and (3) oocyte development and size.

4.3 Materials and methods

4.3.1 Study site and sampling regime

This study was undertaken at Christmas Island (10°30'S, 105°40'E; CI), an oceanic island located approximately 300 km south of Java, Indonesia, and on the Indo-Pacific biogeographic border, which is a hot spot for hybridisation. In this area, Indian and Pacific Ocean species come in secondary contact after being separated during the Pleistocene sea level changes (Hobbs et al. 2009, 2010). Recently, 15 fish hybrids have been reported at CI, and two of them (Genus: *Chaetodon* and *Cephalopholis*) have been assessed for fitness (Payet et al. 2016; Montanari et al. 2017).

Three species of Pomacanthidae from the genus *Centropyge* (i.e. *Centropyge flavissima*, *C. eibli* and *C. vrolikii*), and their hybrids were collected in September 2015 and April 2016 from CI. Since CI is exposed to south-easterly trade winds and southerly swell for most the year (Richards and Hobbs 2014), sampling was restricted to the more sheltered north coasts of the island at depths of 5 to 25 m during both sampling trips. A large size range of each species (*C. flavissima* n=66; *C. eibli* n=47; *C. vrolikii* n=29) and the hybrids (n=34) was targeted using spear (on SCUBA). Hybrids were identified based on their coloration (see chapter 3 for details). For each fish, the total length (TL) was measured to the nearest 1 mm, and the two sagittal otoliths were removed and stored dry in Eppendorf vials. The gonads of each fish were removed and preserved using a fixative containing 4% formaldehyde, 5% acetic acid and 1.3% calcium chloride for 24 hours, then transferred to ethanol prior to histology.

4.3.2 Otolith preparation and reading

Thin otolith sections were prepared using the methods previously described by Wakefield et al. (2010, 2017a). A sagittal otolith (left or right) from each fish was embedded in epoxy resin and sectioned transversely through its primordium in a direction perpendicular to the sulcus acusticus, using a low speed saw (Isomet™ Buehler Ltd) with a diamond tipped blade. Prior to sectioning, the micrometer on each saw was calibrated to improve the consistency in the thickness of otolith sections. Otolith sections were cut thin (i.e. ~0.2 mm), rinsed in 2% hydrochloric acid for ~30 seconds (see Gauldie et al. 1990), and mounted on a glass slide with a cover slip using casting resin. Opaque zones in otolith sections were counted using a dissecting microscope (with 20x stereoscopic oculars) under reflected light at 10-30x magnification, and without any knowledge of the size of fish. All otolith sections were examined independently by two readers (FV and CBW) and the precision between readers was compared using age-bias plots and the Index of Average Percent Error (IAPE, Beamish and Fournier 1981; Wakefield et al. 2017a). The final age for each fish was based on the agreement between two readers.

4.3.3   Gonads histological examination and maturity estimates

Preserved gonads were embedded in paraffin wax, sectioned transversally at ~5 μm, mounted on glass slides and stained with Harris's haemotoxylin and Young's eosin–erythrosine (Woods and Ellis 1994). Ovarian development was assessed microscopically from the identification of the most developed oocytes, and other relevant structures, including the structural appearance of lamellae, thickness of the ovarian wall, intra-lamellar stromal tissue, atretic oocytes, brown bodies and postovulatory follicles. To determine whether each of the angelfish species and hybrid had determinate or indeterminate fecundity, the frequency distributions of oocyte sizes were analysed from ten mature female gonads of each species. Circumferences of 30 randomly selected oocytes (sectioned through their nuclei) from each gonad were measured and used to calculate an oocyte area using imaging software (ImageJ).

Protogynous (female to male) sex change has been widely reported for angelfishes (Moyer et al. 1983) including *Centropyge* spp. (Moyer and Zaiser 1984). During microscopic examination, gonads were classified as being in transition between ovary and testis, i.e. transitional, where degenerating ovarian tissue or oocytes were present and

only male germ cell stages less developed than spermatozoa were present. This was consistent with an assumption that the presence of spermatozoa was indicative of a mature testis. Confirmation of suspected protogynous sex transition was based on the detection of vestigial ovarian structure and the presence of brown bodies or atretic vitellogenic oocytes in testes, or identifying successive stages of protogynous sex transition (see Sadovy and Shapiro 1987)

4.3.4 Data analysis

The growth of each angelfish parent species and hybrid was described using the von Bertalanffy growth equation, i.e. $L_t = L_\infty(1 - \exp^{-k(t-t_0)})$; where L_t is the predicted fork length (mm) of fish at age t (years), L_∞ is the asymptotic length (mm) at an infinite age, k is a constant growth coefficient that determines the rate at which L_t approaches L_∞ (year $^{-1}$), and t_0 is the hypothetical age (years) at which fish would have zero length. Growth curves were fitted by minimising the sum of squares, and estimates of L_∞, k and t_0 ($\pm 95\%$ confidence limits, CL) were determined by random resampling with replacement (i.e. bootstrapping) to generate 2000 sets of parameters. The von Bertalanffy growth parameters and lower and upper 95% CLs were calculated as the median, 2.5, and 97.5 percentiles, respectively, of the 2000 bootstrap estimates (e.g. Wakefield et al. 2015a, 2017b). The growth curves for each species and hybrid were compared using a likelihood-ratio test (Cerrato 1990).

The length at which 50% of females of each angelfish species and hybrid attained sexual maturity (L_{50m}) was determined using a reparameterised form of the logistic equation (e.g. Hesp et al. 2004; Wakefield et al. 2007), i.e.

$$P_L = \left\{1 + \exp\left[-\log_e(19)(L - L_{50m})/(L_{95m} - L_{50m})\right]\right\}^{-1},$$

where P_L is the proportion of mature individuals of each species at a particular fork length L (mm), and the L_{50m} and L_{95m} are the estimated fork lengths (mm) at which 50% and 95% of individuals of each species attained sexual maturity, respectively. This logistic equation was fitted by maximising the sum of the log-likelihoods such that, on the basis of its L, an immature fish would have a likelihood of P_L and a mature fish a likelihood of $1 - P_L$. Values of the L_{50m} and L_{95m} were determined by random resampling with replacement (i.e. bootstrapping) for each dataset to generate 2000 estimates. These

parameters and 95% CLs for the logistic equation were calculated as the median, 2.5, and 97.5 percentiles of the 2000 bootstrap estimates for the proportions of fish that are predicted to be mature for each length class. Estimates of the length at which each angelfish species and hybrid changed sex were calculated using the same equation and approach, but with the lengths at which 50% (L_{50sc}) and 95% (L_{95sc}) of individuals predicted to have changed from female to male for these protogynous hermaphroditic species (Wakefield et al. 2013, 2015b).

4.4 Results

4.4.1 Age and growth

All sectioned otoliths, except those from very small fish (<30 mm), presented annual growth increments visible as alternating translucent and opaque zones when viewed under reflected light. A series of one translucent and one opaque zone was considered to be 12 months of growth, following a previous angelfish age validation study (Choat et al. 2009). The IAPE between the counts of annuli from two readers was 5.1, 3.7, 3.2 and 3.3 for *C. flavissima*, *C. eibli*, *C. vrolikii*, and the hybrid *C. flavissima × C. eibli*, respectively. The annuli count from the two readers was consistent for each species and hybrid and did not differ by more than two years.

All parent species and the hybrid showed rapid growth during the first three years of life, followed by slower growth up to their asymptotic length, which was attained between 6 (*C. eibli*) and 8 years of age (Fig. 4.1). The von Bertalanffy growth parameters k and L_∞ were not significantly different between *C. flavissima* and the hybrid (i.e. *C. flavissima × C. eibli*), but did differ significantly with those of *C. eibli* (P < 0.05). The growth trajectories exhibited by *C. flavissima* and the hybrid were very similar, with both species reaching a larger length at age than *C. eibli* at ≥ five years of age (Table 4.1, Figure 4.2). *C. eibli* had the highest growth coefficient (k=0.54 year^{-1}), followed by *C. vrolikii* (0.47), the hybrid, and *C. flavissima*, which showed similar growth coefficients (0.39 and 0.36 respectively) (Table 4.1, Figure 4.2). Conversely, *C. eibli* exhibited the lowest asymptotic length (95mm) followed by *C. vrolikii* (100), the hybrid (102), and *C. flavissima* (103) (Table 4.1).

The maximum age of the hybrid (12 years) was slightly lower than both the parent species *C. flavissima* (16 years) and *C. eibli* (14 years), but similar to a sympatric congener (i.e. maximum age of 12.5 years for *C. vrolikii*). These small variations in longevity among the three species and hybrid may be an artefact of small sample sizes.
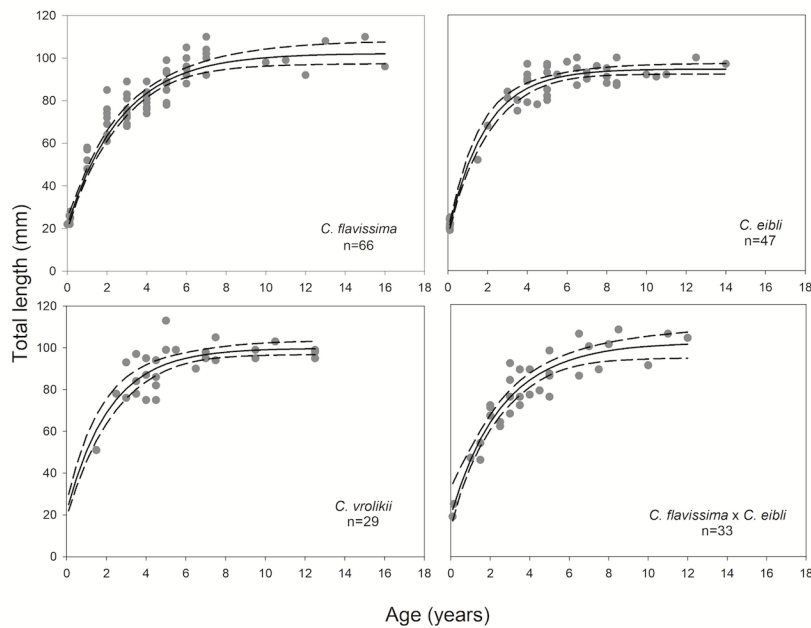
Figure 4.1 von Bertalanffy growth curves (± 95% confidence limits) fitted to the lengths at age of the three angelfish parent species *Centropyge flavissima*, *C. eibli*, *C. vrolikii* and the hybrid *C. flavissima* × *C. eibli* from Christmas Island. Males and females are combined (grey circles), the sample size is indicated under the species names.
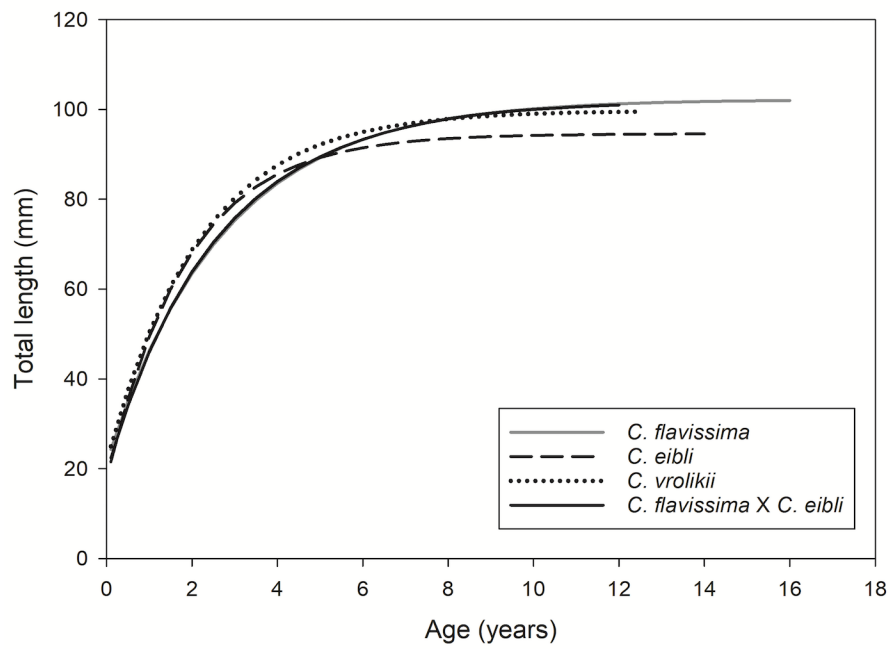
Figure 4.2 Comparison of von Bertalanffy growth curves for each parent species and hybrid.

Table 4.1 von Bertalanffy growth parameters (estimates and associated 95% upper and lower confidence limits) for curves fitted to the total lengths (TL) at age of the three angelfish parent species and the hybrid *C. flavissima* × *C. eibli* in Christmas Island. TL_∞, hypothetical asymptotic length at an infinite age; k, growth coefficient; t_0, hypothetical age at zero length; A_{max}, maximum age; L_{max}, maximum length; n, sample size; r^2, coefficient of determination.

	TL_∞ (mm)	k (yr^{-1})	t_0 (yr)	A_{max}	L_{max}	n	r^2
C. flavissima							
Estimate	103	0.36	-0.65	16.0	110	66	0.93
Upper	108	0.42	-0.53				
Lower	97	0.30	-0.87				
C. eibli							
Estimate	95	0.54	-0.37	14	100	47	0.98
Upper	97	0.64	-0.31				
Lower	93	0.45	-0.47				
C. vrolikii							
Estimate	100	0.47	-0.52	12.5	113	29	0.24
Upper	103	0.59	-0.52				
Lower	97	0.39	-0.52				
Hybrid							
Estimate	102	0.39	-0.55	12	108	34	0.87
Upper	109	0.51	-0.34				
Lower	95	0.24	-1.70				

Table 4.2 Likelihood ratio test results comparing L_∞, k and t_0 across species and hybrids

	Factor	Chi square	df	p
C. flavissima vs C. eibli				
	L_∞	8.30	1	**0.004**
	k	8.62	1	**0.003**
Hybrid vs C. eibli				
	L_∞	7.13	1	**0.008**
	k	4.50	1	**0.034**
	t_0	0.84	1	0.359
C. flavissima vs Hybrid				
	L_∞	0.04	1	0.841
	k	0.16	1	0.689
	t_0	0.18	1	0.671
C. flavissima vs C. vrolikii				
	L_∞	0.87	1	0.351
	k	6.27	1	**0.012**
	t_0	5.12	1	**0.024**
C. eibli vs C. vrolikii				
	L_∞	1.78	1	0.182
	k	2.90	1	0.089
	t_0	4.83	1	**0.028**

4.4.2 Reproduction

Mature gonads were recorded for both females and males of the hybrid *C. flavissima* × *C. eibli* and they exhibited similar development to the parent species (Fig. 4.3). Mature female gonads from parent species and hybrids contained post-ovulatory follicles (Fig. 4.3), indicating the possible spawning activity of the hybrid. The length at which 50% of fish had reached maturity (L_{m50}), and changed sex from female to male (L_{sc50}), did not differ between the parent species and hybrid (Figs 4.4). Size at sex change (L_{sc50}) for the parent species and hybrids was 87.1 mm (TL) for *C. flavissima*, 86.9 mm for *C. eibli*, 89.4 mm for *C. flavissima* × *C. eibli*, and 93.6 mm for *C. vrolikii*. The proportions of mature fish increased with fish size (Fig. 4.4), with 50% (L_{m50}) of individuals from *C. flavissima*, *C. eibli* and the hybrid attaining maturity at similar sizes: 69.1, 61.2 and 63.7

mm TL, respectively. Due to the low number of samples, this analysis could not be performed on the less abundant parent species *C. vrolikii*.

The size distribution of oocytes of the hybrid *C. flavissima* × *C. eibli* was similar to that of the parent species (Fig. 4.5). The majority of the oocytes from the two parent species *C. flavissima* and *C. eibli*, and the hybrid, measured between 0.02 and 0.04 mm. The wide distribution but lower frequency of larger oocytes ($>0.06mm^2$) suggests continued development of oocytes over the reproductive period and indeterminate reproduction (e.g. Hyndes et al. 1996).
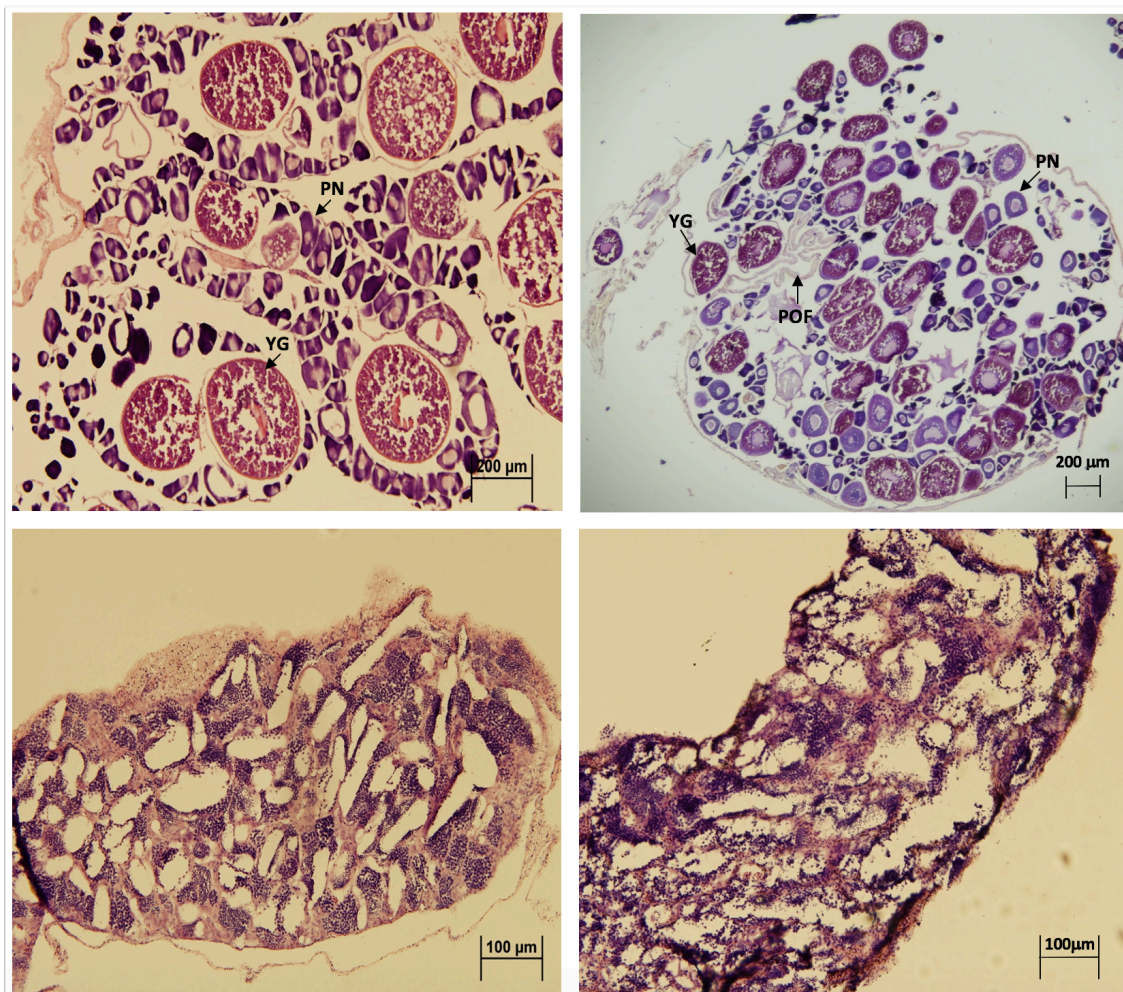
Figure 4.3 Histological sections showing different stages of oocytes and gametocytes from the parent species *C. flavissima* (left) and the hybrid *C. flavissima* × *C. eibli* (right). The top photos show typical mature ovaries, with perinuclear (pn) and yolk-granule (yg) oocytes, and post-ovulatory follicles (POF).
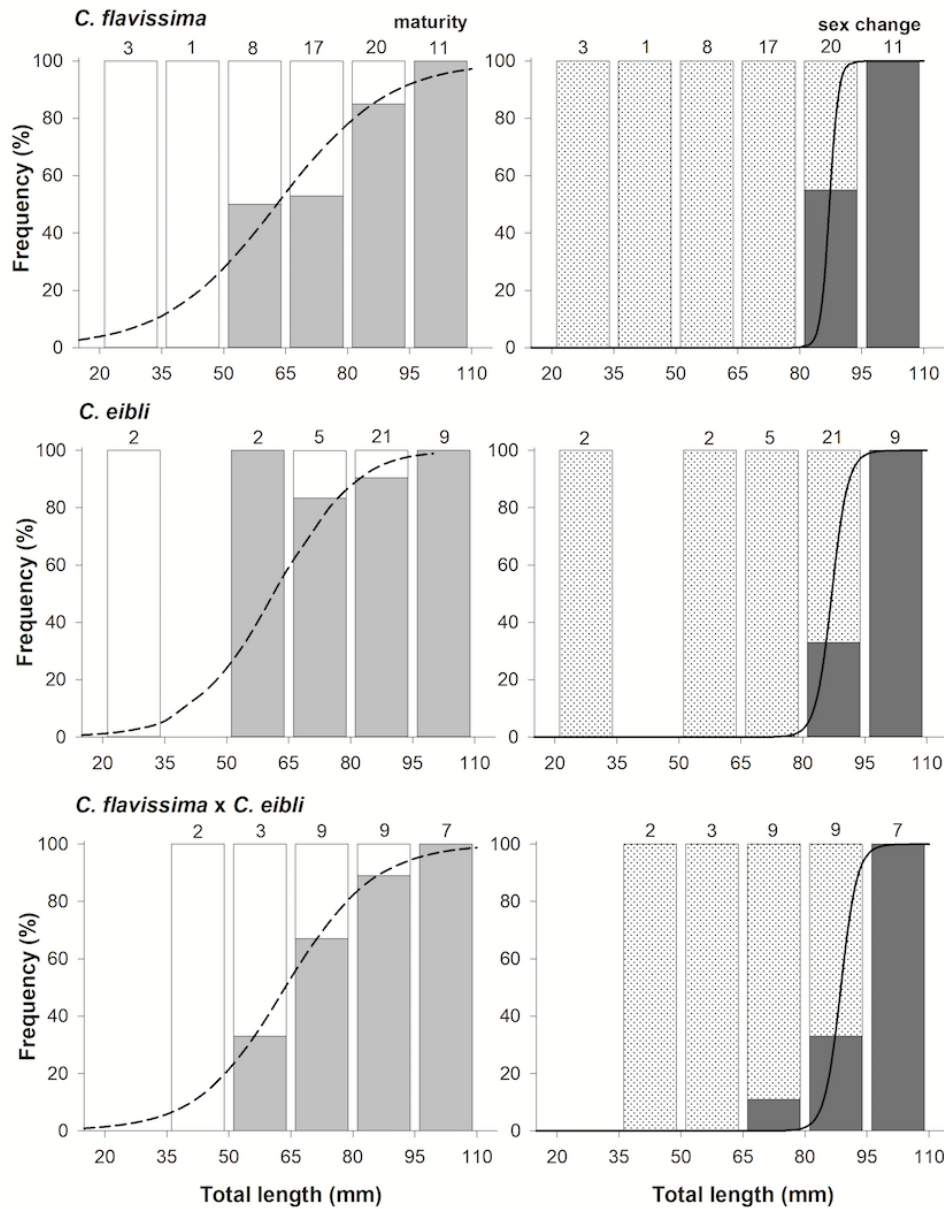
Figure 4.4 Percentage frequency of immature (white bars) and mature (light grey bars, mature females and males combined) individuals (left) and female (dotted bars) and male (dark grey bars) individuals (right) in sequential 15 mm TL size classes (sample sizes shown) for the two angelfish parent species *Centropyge flavissima*, *C. eibli* and the hybrid of *C. flavissima* × *C. eibli*. Logistic curves were derived from the probability that a fish of a given TL is mature (dashed lines) or male (solid lines).
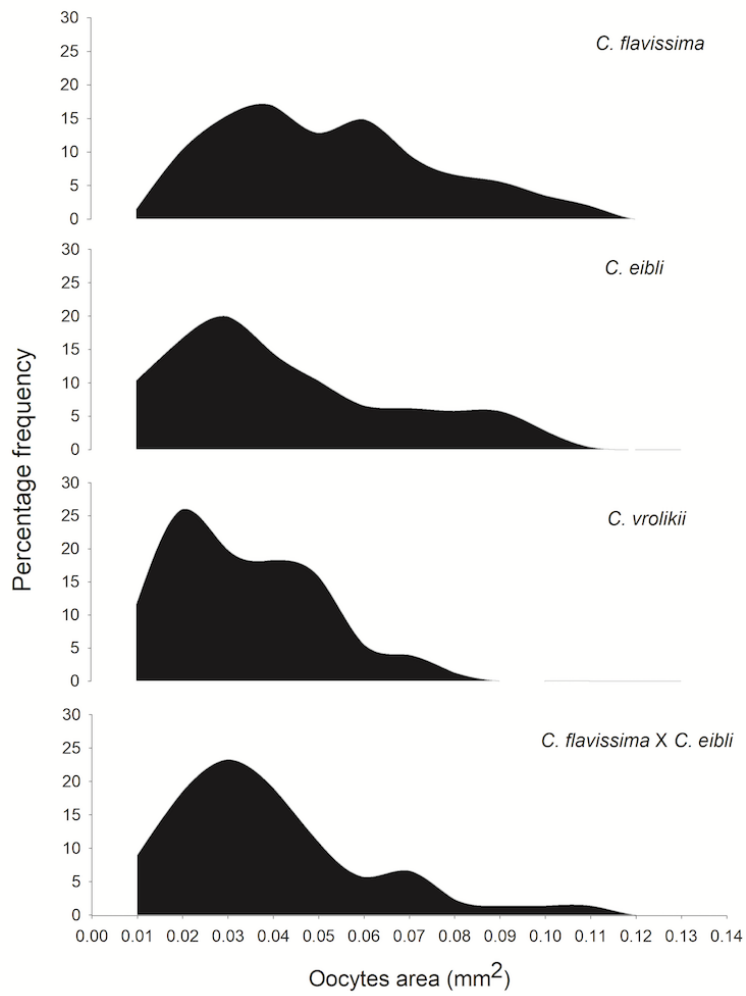
Figure 4.5 Percentage frequencies for the oocyte areas from the ovaries of each angelfish parent species (*C. flavissima*, *C. eibli* and *C. vrolikii*) and hybrids (*C. flavissima* × *C. eibli*). Thirty oocytes were measured in 10 mature female fishes for each species/hybrid, the size range of the fishes was from 60 to 90 mm.

Table 4.3 Total lengths (mm TL) and ages (years) at which 50 and 95% (L_{50}, L_{95}, A_{50} and A_{95}, respectively) of the two protogynous hermaphroditic angelfish parent species and their hybrid reach sexual maturity and change sex (i.e. from female to male) at Christmas Island, together with the life history invariant relationship between maturity and sex change with asymptotic length (L_{m50}/L_∞ and L_{sc50}/L_∞), and the minimum length (TL) and age (years) recorded for mature and male fish (L_{min} and A_{min}).

	Maturity							
	L_{50}	L_{95}	L_{m50}/L_∞	L_{min}	A_{50}	A_{95}	A_{min}	n
C. flavissima								
Estimate	62.8	101.7	0.67	48	1.9	14	1.5	60
Upper	73	120.2			2.7	-		
Lower	48.5	89.6			1.1	4.9		
C. eibli								
Estimate	61.3	87.5	0.64	52	1.5	4.5	1.5	39
Upper	78.6	102.4			2.9	-		
Lower	32.5	40.9			0.4	0.6		
Hybrid								
Estimate	63.5	94.9	0.62	62	1.9	7.3	2.5	30
Upper	75.6	121.1			2.9	-		
Lower	44.9	77.1			0.9	3		
	Sex Change							
	L_{50}	L_{95}	L_{sc50}/L_∞	L_{min}	A_{50}	A_{95}	A_{min}	n
C. flavissima								
Estimate	87.2	90.3	0.85	85	4.4	5.0	2.5	60
Upper	89.0	93.8			4.8	5.9		
Lower	84.7	85.7			4.0	4.2		
C. eibli								
Estimate	87.0	92.7	0.92	87	4.4	7.8	6.5	39
Upper	89.1	98.7			5.1	-		
Lower	85.2	86.3			4.0	4.2		
Hybrid								
Estimate	88.7	94.5	0.87	68	4.8	7.0	3	30
Upper	98.2	106.1			-	-		
Lower	80.0	81.2			3.3	3.5		

4.5 Discussion

Hybrids usually display lower level of fitness compared to parent species in terrestrial systems (Arnold and Martin 2010). However, angelfish hybrids from Christmas Island showed similar fitness to one or both parent species in growth and reproductive characteristics, respectively. Although these parameters are considered the most appropriate measures of fitness for marine fish (Payet et al. 2016; Montanari et al. 2017), different parameters, such as tolerance to abiotic environments, fertility, susceptibility to predation, and metabolic capacities (Arnold and Hodges 1995), has been used in terrestrial studies. Thus, the use of different parameters representing fitness in marine versus terrestrial systems may influence the conclusions made regarding hybrid fitness in the two environments.

Angelfish hybrids at CI showed a similar growth rate and maximum size to the larger of the parent species *C. flavissima*, but grew less rapidly and to a larger size compared to the smaller parent species *C. eibli*. In comparison, the hybrids exhibited a shorter life span than both parent species, especially *C. flavissima* (maximum age 12 vs 16 years). Hybrids of butterflyfishes (*Chaetodon*) and groupers (*Cephalopholis*) also exhibit similar growth rates to their parent species (Payet et al. 2016; Montanari et al. 2017), but grouper hybrids live longer than their parent species (Payet et al. 2016). This can play a positive role on the reproductive output of hybrids, as a mature fish that lives longer has a greater lifetime reproductive success. In the case of angelfish, the lower longevity in hybrids may have been due to smaller sample sizes as a consequence of their lower abundances (Vitelli et al. unpublished). It is, therefore, possible that the longevity of hybrids was similar to that of the parent species.

Despite the smaller body size of *C. eibli*, both parent species and the hybrid reached maturity and changed sex at similar sizes. Maturity is reached at ~ 61 mm TL and sex change from female to male occurred at ~90 mm TL in both parent species and the hybrid. These two traits, combined with the length, age and growth of the fish, are crucial factors influencing the reproductive output in haremic fish (Warner 1984; Moyer and Zaiser 1984). However, *C. eibli* grows slightly faster than the hybrid and *C. flavissima*. It is, therefore, possible that *C. eibli* reaches maturity and changes sex at an earlier age than

the other parent species and hybrid, resulting in greater reproductive success for this species.

Angelfishes from the genus *Centropyge* are protogynous hermaphrodites and haremic (Allen et al. 1998, Kuiter et al. 2003), with their harems typically comprising 2-5 fishes where the biggest fish is the male and the other fish are females (Allen et al. 1998). Thus, individual fish that grow faster and reach a larger size have a greater chance at becoming the male in the harem. Becoming a male is important because the reproductive success of a male is equal to the combined total reproductive success of all the adult females in the harem. Since we found that hybrids (*C. flavissima* × *C. eibli*) have the potential to grow as fast and as large as the abundant parent species *C. flavissima*, they are likely to have the same chances of becoming the male in the harem, and achieve levels of reproductive success similar to that of the parent species, *C. flavissima*. In comparison, *C. eibli* grows faster than *C. flavissima* and the hybrid during the first five years of life, with the potential to mature earlier and thereby reproducing for a longer period over its life span. However, since the asymptotic length of *C. eibli* was the lowest (95 vs 100 and 103 mm), and fecundity is related to body size (Warner 1984), reproductive output overall may not be greater. In addition, since larger fish outcompete smaller fish to become the dominant male in a harem (Moyer et al. 1983; Warner 1984), the smaller size of *C. eibli* would reduce its chances of becoming the dominant male in the harems compared to the larger *C. flavissima* and hybrid. Similar results have been shown for groupers from the genus *Cephalopholis*, which exhibit the same haremic reproductive system and whose hybrids showed the same size-biased pattern of sexual development as their parent species (Payet et al. 2016).

The reproductive viability of *Centropyge* hybrids also appeared to be similar. The hybrids exhibited similar gonadal ontogeny to their parent species, with no differences in the structure of the gonads or size and development range of oocytes. Previtellogenic to mature stage oocytes were present in mature ovaries of hybrids and parent species. Post-ovulatory follicles (POF) were also present, indicating the ability of the hybrids to release mature eggs into the water to spawn. Hybrids have similar spawning patterns to the parent species, and the parent species court and breed with the hybrids (Hobbs et al. 2018). Collectively, this shows that hybrids can produce mature gametes and release these gametes when spawning with other individuals. We recognise that this does not confirm

the viability of hybrid gametes, however, molecular and photographic evidence of backcrossed individuals and introgression between the parent species (DiBattista et al. 2016b; Appendices 4.1 and 4.2) confirm that hybrids are fertile and gametes are viable. Similarly, butterflyfishes that hybridise at CI had fully developed gonads that are viable as demonstrated through genetic studies (Montanari et al. 2017). Given the hybrids' similar reproductive potential to the most abundant parent species *C. flavissima*, it is interesting how hybrids still remain less abundant. This study did not investigate crucial reproductive factors such as fecundity of the different species and survival rates of the larvae, which could have a great impact on each species' abundances. Thus, further studies are needed to be able to answer this question.

Hybrid angelfishes showed similar levels of fitness in terms of growth and reproductive output, but heterosis (decreased fitness), or hybrid vigour (increased fitness) may be expressed in other attributes or behaviours not considered in this study. For example, hybrids could have the capacity to inhabit new niches or adapt to changing environmental conditions (Seehausen 2004; Karrenberg et al. 2006). However, long term (14 years) ecological surveys on angelfish hybrids at CI (Vitelli et al. unpublished) showed a high niche overlap in terms of habitat and food resources between hybrids and parent species, suggesting that hybrids use similar niches to parent species. This is not surprising, as niche overlap is considered one of the most important factors promoting hybridisation (Frisch and van Herwerden 2006; Camp et al. 2016; Montanari et al. 2016). However, other attributes could differentiate them from their parent species. Furthermore, due to genetic recombination, hybrids may be better adapted to future environment conditions. Even though involving closely-related species, hybridisation may have important evolutionary consequences because the unique genetic combinations of hybrids may enable them to succeed in future environmental conditions. Furthermore, introgression may allow transfer of favorable genes into the parent species.

Chapter 5. Social structure of hybridising angelfishes at Christmas Island

5.1 Abstract

Hybridisation can have important evolutionary outcomes, mainly depending on the fitness of the hybrids and whether they are socially accepted by other individuals and able to reproduce. It is, therefore, important to investigate their social status, such as their ability to reach a high rank in the mating groups for species that form harems (Montanari et al. 2016). In this study, we investigated the social structure of hybridising angelfishes (*Centropyge flavissima*, *C. eibli*, and *C. vrolikii*) at Christmas Island. *Centropyge flavissima* was the only species that formed monospecific harems, whereas *C. eibli* and *C. vrolikii,* were found only in heterospecific harems with *C. flavissima*, but not with each other. Hybrids most commonly formed harems with *C. flavissima*, which was the most abundant of the three parent species, followed by harems with mixed species *C. flavissima + C. eibli*, and *C. flavissima + C. vrolikii*. There was no significant difference in either total number of fish or number of males between pure *C. flavissima* harems, heterospecific harems with no hybrids and harems containing hybrids. In general, rank number one was mainly occupied by *C. flavissima* (56.8%), followed by hybrids (32.6%), *C. eibli* (7.4%), and *C. vrolikii* (3.2%). The ranks of the fish were positively related with the size of the fish, with the bigger size corresponding with the highest rank in the harem. The size-at-rank did not differ significantly between parent species and hybrids. This study supports the theory that, due to their high fitness and reproductive potential, hybrids are likely to persist at Christmas Island.

5.2 Introduction

Hybridisation has long been considered an important process that can shape the evolution of plants and animals (Stebbins 1959; Arnold 1997). The potential evolutionary consequences of this process highly depend on the fitness of the hybrids and on their capacity to reproduce and to be socially accepted by their parent species (Arnold and Hodges 1995; Arnold and Martin 2010). Based on the hybrids' fitness and ability to reproduce, hybridisation could produce different evolutionary scenarios (Arnold and Hodges 1995; Arnold and Martin 2010; Richard and Hobbs 2015). For example, hybrids

could display lower fitness and reproductive success compared to parent species, or, in the worst scenario be sterile, meaning an evolutionary 'dead end' (Barton 2001). On the contrary, hybrids could show a high level of fitness and, through genetic recombination, evolve into a new species that could colonise new habitats or be able to adapt to challenging environmental conditions (Lewontin and Birch 1966; Arnold 1997).

The fitness of an individual is usually measured by its capability to reproduce and transmit its gene pool to future generations, which has been considered vital for the evolution of the species (Mayr 2002). In the terrestrial environment, fitness has been measured using biological parameters such as hybrids' susceptibility to predation, tolerance to abiotic environments, and fertility (Arnold and Hodges 1995). In general, the fastest an individual grows and matures, the greater its chances to be reproductively successful (Brommer 2000; Mayr 2002). However, such parameters will only reveal part of the critical information on hybrids' reproductive potential. To fully evaluate hybrid fitness, it is equally important to investigate their social status, such as their ability to reach a high rank in the mating groups for species that form harems (Montanari et al. 2016). Social conditions, such as the size of the mating groups/harems and the rank of an individual, have been shown to be an important factor influencing the lifetime reproductive success of both terrestrial and marine animals (Cowlishaw and Dunbar 1991; Modig 1996; Gainsford et al. 2015). In general, larger social groups or harems, and a higher rank, are usually positively related to high reproductive success (Cowlishaw and Dunbar 1991). For example, elephant seals with bigger harems showed to have higher mating success (Modig 1996).

Recent studies have showed that hybridisation is a common process in marine environments, especially in corals and coral-reef fishes (Hobbs and Allen 2014; Richards and Hobbs 2015). However, only a few studies have been carried out on the fitness of marine fish hybrids, which has been measured through biological and histological analyses investigating growth rates and maturity/fertility (Payet et al. 2016; Montanari et al. 2018, Chapter 4). For example, hybrids of butterflyfishes (family *Chaetodonthidae*), groupers (genus *Cephalopholis*), and angelfishes (genus *Centropyge*) exhibit similar fitness (e.g. growth and maturity) to their parent species and are able to reproduce (Payet et al. 2016; Montanari et al. 2018, Chapter 4). However, no study on the social status of

coral-reef fish hybrids has yet been done, precluding important information on possible evolutionary consequences of hybridisation in coral-reef fishes.

Three species of angelfishes from the genus *Centropyge* (*C. flavissima*, *C. eibli*, and *C. vrolikii*) have been reported to form mixed harems and hybridise at Christmas Island (Hobbs and Allen 2014). These species are haremic and exhibit protogynous hermaphroditism (Moyer et al. 1983; Moyer and Zaiser 1984). Harems are usually composed of a larger dominant male and two to six females, with the male spawning at dusk with each of the females of the group. If the male is removed from the harem, the biggest female changes sex and becomes the dominant male (Kuiter et al. 2003; Moyer and Zaiser 1984). Thus, being a male (rank number one) in a larger harem will translate to higher reproductive success. In the previous chapter, the fitness of the hybrids was evaluated in terms of their growth rates and ability to mature and spawn. Angelfish hybrids are able to grow and mature as fast as their parent species and display fully developed gonads (Chapter 4). However, to fully evaluate their reproductive success, and therefore, the possible evolutionary consequences of angelfish hybridisation, it is equally important to investigate the social status (rank) of the hybrids and their ability to be accepted into the harems and mate.

The general aim of this study was to evaluate the social condition of angelfish hybrids, and to determine their potential reproductive success compared to their parent species. To achieve this, we have determined: (1) whether parent species are accepting hybrids in the harems, and if so, in what proportions; (2) whether the size and sex composition of heterospecific and homospecific harems are similar; and (3) whether the social hierarchical rankings of the hybrids are similar to their parent species in heterospecific harems, and whether body size is positively related with the rank.

5.3 Methods

5.3.1 Study site and sampling regime

This study was carried in September 2015 and April 2016 at Christmas Island (10°30'S, 105°40'E), an oceanic island located approximately 300 km south of Java, Indonesia. Due to south-easterly trade winds and southerly swell for most the year (Hobbs et al. 2014), underwater observations and sampling were restricted to the more sheltered north coasts

of the island at depths of 5 to 25 m. Underwater observations were conducted to characterize the composition of social groups (harems) formed by the three species of angelfish (*Centropyge flavissima, C. vrolikii* and *C. eibli*), and their hybrids at eight sites.

5.3.2 Abundance

To determine the relative abundances of the three parent species (*Centropyge flavissima*, *C. eibli*, and *C. vrolikii*) and their hybrids, the density of each angelfish species and its hybrids were estimated by underwater visual censuses on ten separate occasions over 14 years (2002-2015) as outlined in Chapter 3 (See Chapter 3 for details).

5.3.3 Harems composition

During sampling in 2015 and 2016, a total of 60 pure *C. flavissima* harems, 60 mixed species harems without hybrids, and 60 harems containing at least one hybrid were randomly selected at those eight sites. Hybrids were identified based on their colouration being a mix between the two parent species colours and patterns (see Appendix 3.1). Each harem was observed and described in terms of species composition and rank of each fish present in the harem. To characterize the composition of harems, each social group forming a harem was observed for approximately 5 min. During this time, the species or hybrid type (based on coloration), and rank of each individual within the social group was recorded. In addition, any aggressive or chasing behaviour towards the hybrids (if any) was recorded. The rank of each fish was determined based on the size (TL) of the fish, with the bigger fish having the higher rank in the harem (Moyer et al. 1983). To determine the sexual composition of the harems, 63 harems were selected based on their species composition (11 pure *C. flavissima*, 36 mixed without hybrids, and 16 mixed with hybrids), and all fishes from each harem were collected using spear (on SCUBA), and measured (total length (TL) to the nearest 0.1mm. To determine the sex of each fish, the gonads of each fish were removed and preserved using a fixative containing 4% formaldehyde, 5% acetic acid and 1.3% calcium chloride for 24 hours, then transferred to ethanol prior to histology.

5.3.4 Histology

Preserved gonads were embedded in paraffin wax, sectioned transversally at ~5 µm, mounted on glass slides and stained with Harris haemotoxylin and Young's eosin–

erythrosine (Woods and Ellis 1994). Ovarian development was assessed microscopically from the identification of the most developed oocytes, and other relevant structures, including the structural appearance of lamellae, thickness of the ovarian wall, intra-lamellar stromal tissue, atretic oocytes, brown bodies and postovulatory follicles.

5.3.5 Data analysis

A Chi Square test was run to compare the relative abundances of the 2 rarest parent species (*C. eibli* and *C. vrolikii*) and hybrids (*C. flavissima* × *C. eibli*, *C. flavissima* × *C. vrolikii*, and *C. eibli* × *C. vrolikii* combined) inside mixed harems with the relative abundances of the same species and hybrids across all sampling sites at Christmas Island. The common species *C. flavissima* was not included in this analysis due to its extremely high abundance compared to the other species, suggesting that the presence of *C. eibli* and *C. vrolikii* is likely to influence hybridisation (see Chapter 3).

To test for differences in the size (number of fish) of three types of angelfish harems (pure *C. flavissima*, heterospecific with no hybrids, and hybrids harems), a one-way ANOVA was run, with harem type as a fixed factor.

5.4 Results

5.4.1 Harems composition

Centropyge flavissima formed the only monospecific harems (n=155), whereas *C. eibli* and *C. vrolikii,* were found only in heterospecific harems with *C. flavissima* (n=117), but not with each other (Fig. 5.1). Hybrids were observed mixing in harems and were not being aggressively attacked or excluded from the harem (no aggressive behaviour event was recorded during our observations). The hybrids formed harems with all species and the species composition of the harems reflected the underlying patterns of abundance for the parent species. Hybrids most commonly formed harems with *C. flavissima* (n=61), which was the most abundant of the three parent species, followed by harems with mixed species *C. flavissima* + *C. eibli* (n=17), and *C. flavissima* + *C. vrolikii* (n=6). A small percentage of hybrids was also found forming harems with other hybrids (n=7) and with *C. vrolikii* (n=1) (Fig. 5.1). The mixed harems without hybrids mainly comprised the two most abundant parent species *C. flavissima* + *C. eibli* (n=63), followed by *C. flavissima* + *C. vrolikii* (n=46), and a mix of the three species (n=8) (Fig. 5.1). The percentage

frequency of occurrence of the rarest parent species (*C. eibli* and *C. vrolikii*) and hybrids

(*C. flavissima* × *C. eibli*, *C. flavissima* × *C. vrolikii*, and *C. eibli* × *C. vrolikii* combined)

inside mixed hybrid harems reflected their relative abundances (from UVC surveys) (Fig.

5.2). Chi Square test did not show significant differences (P=0.935).
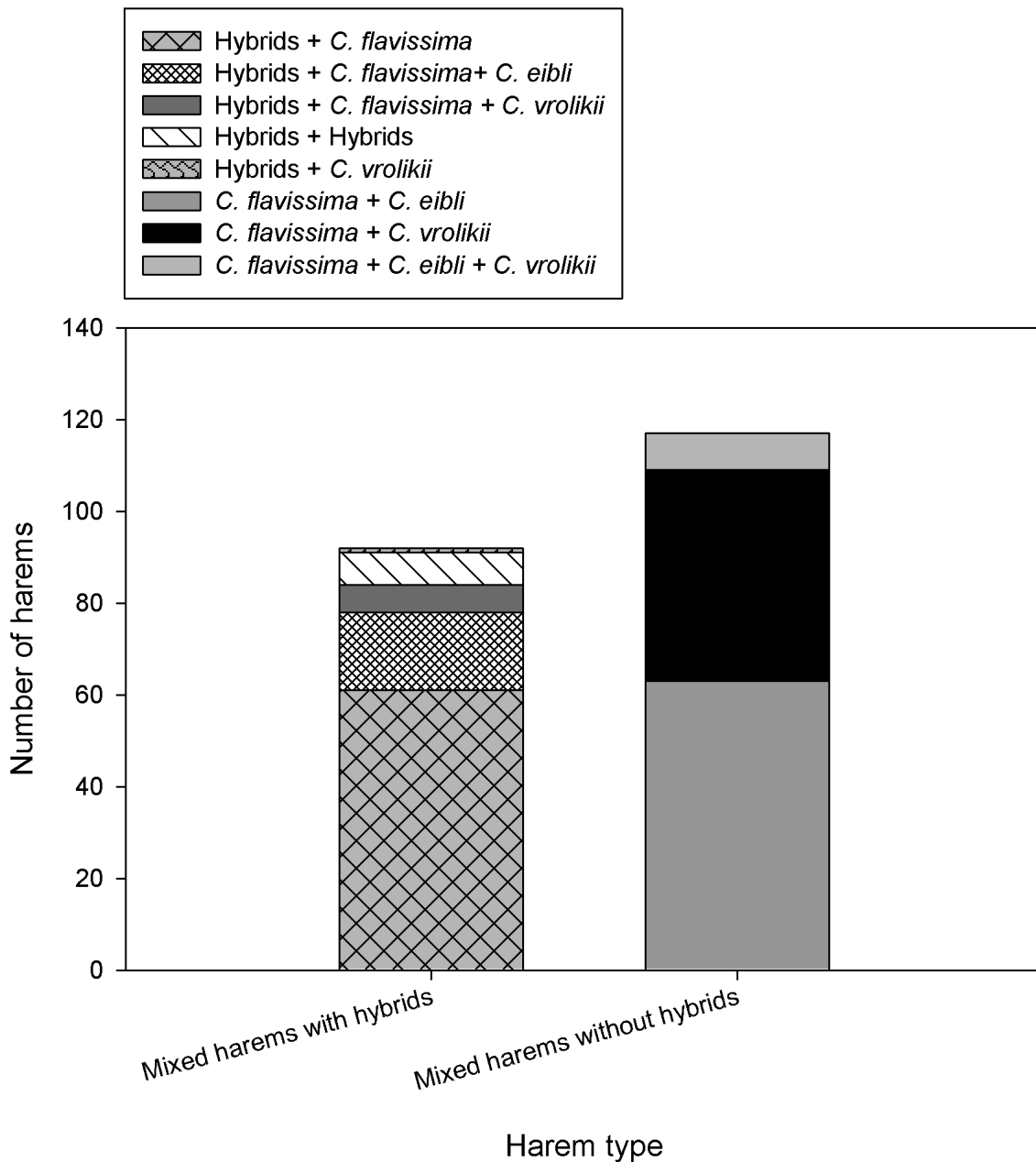
Figure 5.1 The number of mixed harems containing hybrids (*C. flavissima* × *C. eibli*, *C. flavissima* × *C. vrolikii*, and *C. eibli* × *C. vrolikii* combined, left), and the number of

mixed harems containing parent species without hybrids (right). Harem composition was recorded during timed swim transects at Christmas Island.
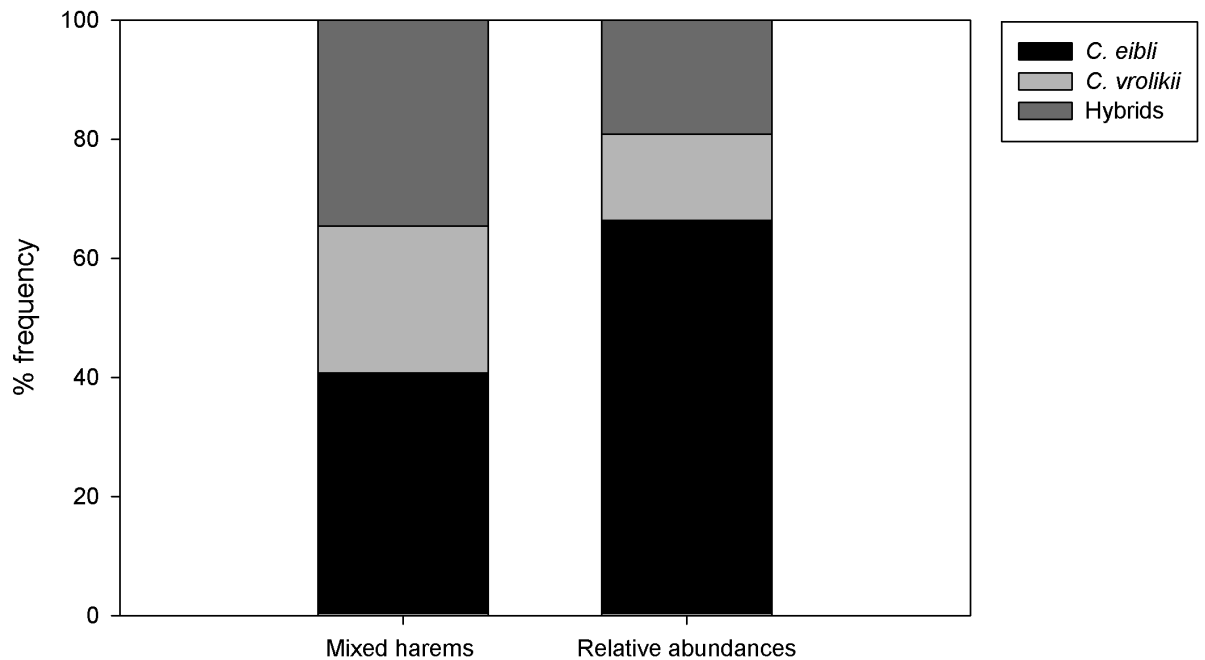
Figure 5.2 Frequency of the 2 rarest parent species and hybrids (*C. flavissima* × *C. eibli*, *C. flavissima* × *C. vrolikii*, and *C. eibli* × *C. vrolikii* combined), present inside mixed harems (left) compared with their relative abundances at Christmas Island (right). Due to its high abundance compared to the other species, *C. flavissima* was not included in the analysis.

5.4.2 Harems size and sex composition

There was no significant difference in either the total number of fish (P=0.286) or the number of males between pure *C. flavissima* harems, heterospecific harems with no hybrids and harems containing hybrids (Table 5.1). Mixed harems without hybrids showed a significantly lower number of females (p<0.01, Table 5.2), compared to mixed harems with hybrids and pure harems of *C. flavissima*. All harems consisted of an average of ~4 fishes, and harems with 2 males were found in each type of harems (Fig. 5.4).
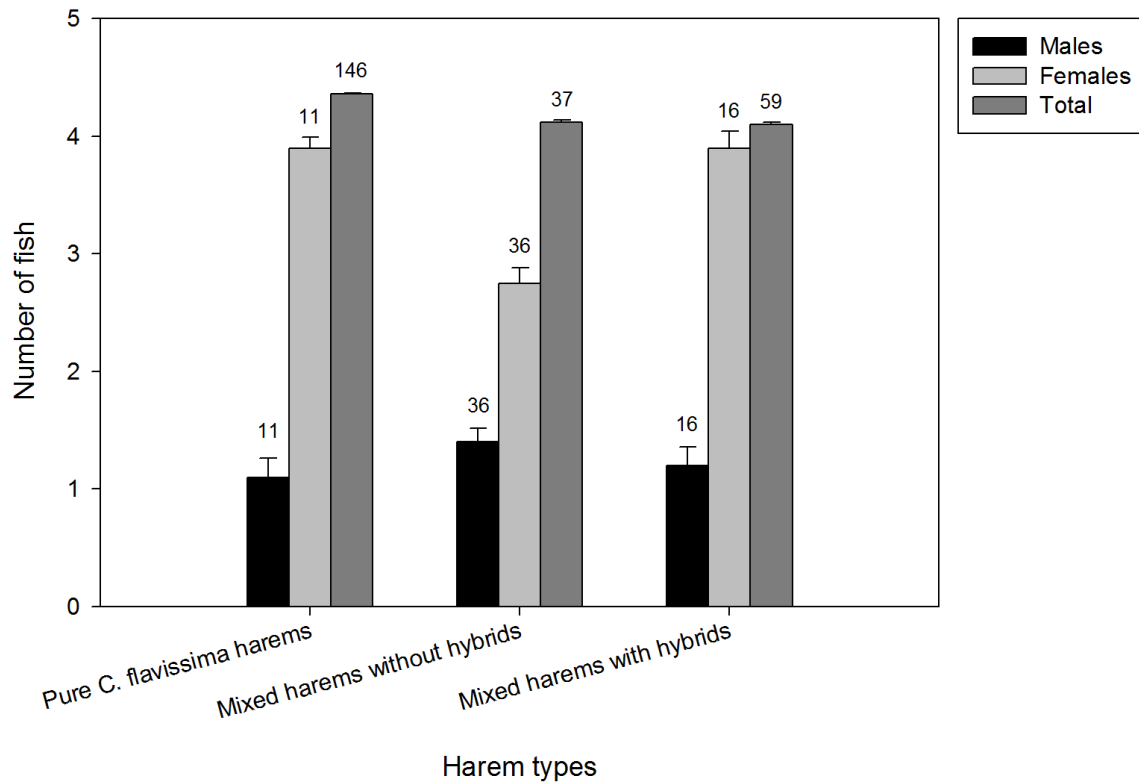
Figure 5.3 The average number (+SE) of males, females, and total fish present in each of the different types of harems. Hybrids are combined (*C. flavissima* × *C. eibli*, *C. flavissima* × *C. vrolikii*, and *C. eibli* × *C. vrolikii*), and sample size is indicated on top of each bar.

Table 5.1 Results of ANOVA testing the number of fish present in the three types of angelfish harems (pure *C. flavissima*, heterospecific with no hybrids, and hybrids harems) at Christmas Island. Harem type was a fixed factor (three levels).

	df	*Mean Square*	*F*	*Sig.*
Total fish	2	1.560	1.259	.286
Males	2	0.477	2.407	.099
Females	2	10.670	10.565	.000

Inside mixed harems with hybrids, one third (32.6%) of the male fish were hybrids (Rank 1, Fig. 5.4). Ranks two, three, four, five and six were occupied by similar percentages of hybrids (~22%). In general, rank number one was mainly occupied by *C. flavissima* (56.8%), followed by hybrids (32.6%), *C. eibli* (7.4%), and *C. vrolikii* (3.2%). Chi Square test showed no significant differences between the frequencies of occurrence of the three angelfish parent species and hybrids in the first three ranks (P=0.213), and between the sex ratio of the hybrids and their expected sex ratio from overall angelfish histological analysis (P=0.496).
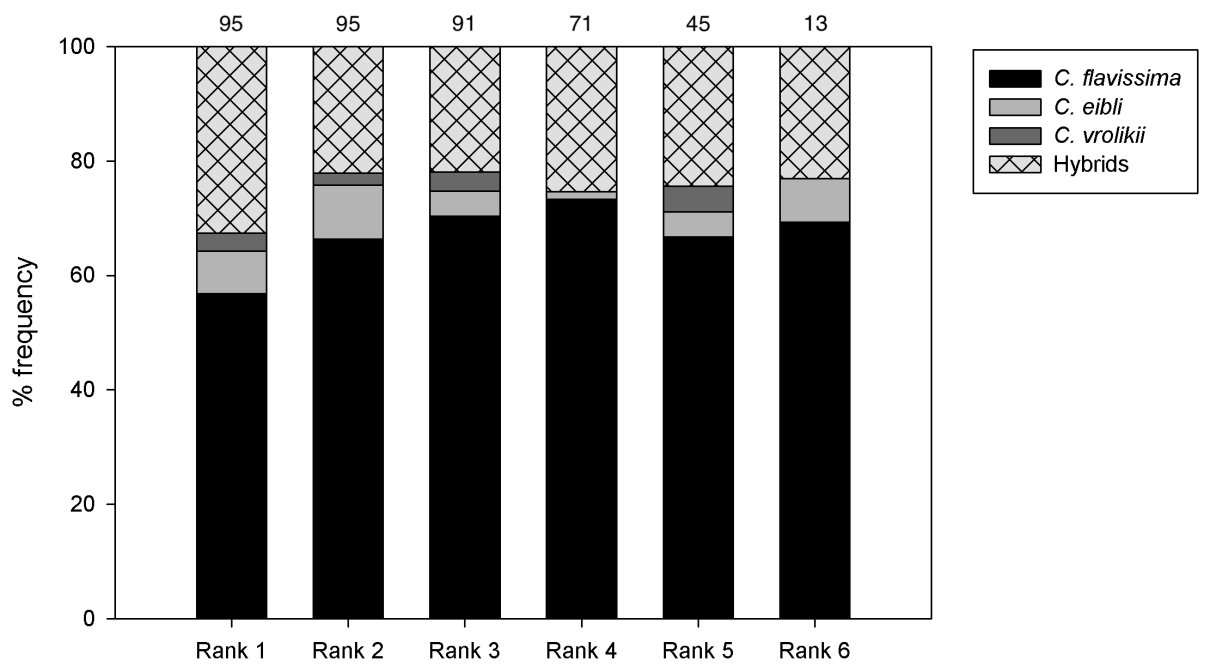
Figure 5.4 Proportion of parent species and hybrids at different rank status in mixed harems at Christmas Island. Hybrids are combined (*C. flavissima* × *C. eibli*, *C. flavissima* × *C. vrolikii*, and *C. eibli* × *C. vrolikii*), and sample size (number of harems) is indicated on top of each bar.

5.5 Discussion

In this study, we showed that hybrids, which have similar fitness to the parent species (Chapter 4), are accepted into mixed harems and were capable of reaching a high rank in those harems. Hybrids were not being aggressively chased by the three parent species, and the proportion of hybrids and two rare species (*C. eibli* and *C. vrolikii*) reflected their relative abundances at Christmas Island. However, a higher proportion of hybrids (~30%) became the dominant male, compared to the other two rare species (*C. eibli* and *C. vrolikii*), suggesting that they may have higher reproductive potential compared to the two rare parent species.

The influence of rarity in forming mixed species harems is evident in the proportion of species in mixed harems. For example, given the very high relative abundances of *C. flavissima*, it is not surprising that this species formed the only monospecific harems, and was present in most of the surveyed harems. In contrast, the rare species, *C. eibli* and *C. vrolikii*, were only found in heterospecific harems and mixed with the other two species and/or hybrids. The species composition of heterospecific harems reflects the actual relative abundances of the two rare species (*C. eibli* and *C. vrolikii*) and hybrids. Harems including *C. flavissima* and *C. eibli* and the hybrid between these two species formed the greatest proportion of heterospecific harems, while harems comprising *C. vrolikii* and the extremely rare hybrid *C. flavissima* × *C. vrolikii* were rare. For haremic species, such as pygmy angelfishes, to be reproductively successful, it is vital for an individual to be accepted into the harem. Similarly, hybrids of coral trout have been reported to actively spawn in mixed haremic social groups at Christmas Island (Payet et al. 2016). Conversely, Wirtz (1999) showed that it is common for animal hybrids to be rejected by parent species, in particular females being more discriminating than males.

The size of the harem or the social group can be another important factor influencing the reproductive success of a species (Modig 1996). More females present in a harem will provide more chances of spawning with a male and passing on their genes to future generations. For example, the size of the harem increases the reproductive success for male elephant seals (Modig 1996). In the case of angelfishes, hybrids in mixed harems showed no differences in number of males, females, and total fish compared to pure harems of *C. flavissima* and mixed harems without hybrids. Thus, when hybrids are

present in mixed harems, they have the same potential to reproduce as their parent species in terms of number of possible partners. However, their reproductive potential is lower than that of *C. flavissima*, which is far more abundant and forms numerous monospecific harems.

In haremic species, becoming the dominant male allows a fish to be more reproductively successful (Moyer and Zaiser 1984; Aldenhoven 1986). Pygmy angelfish harems usually comprise one dominant male which spawns every day at dusk with each of the female members of the harems (Bauer and Bauer 1981). Thus, being a male and occupying rank number one means higher spawning frequencies (Ang and Manica 2010). In mixed harems, hybrids of angelfishes showed a strong ability to become the dominant male, with almost 33% of mixed harems having a hybrid as the dominant male. This was lower than that of the most abundant parent species, *C. flavissima* (56%), but higher than the other two more rare parent species (8% for *C. eibli* and 3% for *C. vrolikii)*. The importance of ranks has been shown in several angelfish species (Moyer et al. 1983; Ang and Manica 2011), and other fish families such as Labridae (Hoffman 1985) and clownfish (Buston 2003). Furthermore, the link between a high rank and a successful reproductive output has been demonstrated in multiple primate species such as the Savanna Baboons (Bercovitch 1986; Cowlishaw and Dunbar 1991). Hybrid pygmy anglefishes are therefore likely to have greater reproductive success than the rare parent species through greater spawning activity of the males.

Body size of the individual can also influence the reproductive success of terrestrial and marine species (Balmford et al. 1992; Downing et al. 1993; Anderson et al. 2012). In marine fish, a larger male may help defend the females in its territory against other aggressive males, and females usually choose larger males as mates, due to their greater reproductive output (Williams 1975; Rakitin et al. 2001). In protogynous, haremic species, such as pygmy angelfishes, it is important for an individual to grow large, as its body size will determine its rank in the harem (Moyer et al. 1983). Here, we showed that hybrids can grow as big as their parent species and occupy rank number one in the harems. The importance of the body size has also been shown in other non-haremic fish families such as salmons (Andreson et al. 2012), and cichlids (Awata et al. 2006; Kohda et al. 2008). For example, Magnhagen and Kvarnemo (1989) showed that female gobiids generally laid their eggs in the nest of bigger males. Body size is important for females

as well, in particular, in protogynous, haremic fish. The biggest female will change sex and become the dominant male 'taking over' the harem (Shapiro 1979; Moyer et al. 1983; Sakai 1997).

Lifetime reproductive success is the currency of fitness (Mills and Beatty 1979). The individual that has the greatest number of offspring (reproductive success) will contribute the most to future generations and their genetics will dominate the population (Mayr 2002). This study adds some vital information on the fitness of hybrids from the genus *Centropyge*, in terms of their social conditions. In a previous study (Chapter 4), we compared the life history of the hybrids to the one of their parent species, and showed that hybrids are as fit as the more abundant species *C. flavissima* in terms of growth and reproductive characteristics. However, life history alone is not enough to evaluate their reproductive success (Montanari et al. 2016). While the hybrids have similar life history characteristics to their parent species, if the they are not able to find a mate and reproduce then they will not have reproductive success. Here, we showed that hybrids are not only accepted into harems by the three parent species, but almost one third of the mixed harems comprised a hybrid as the dominant male, which would lead to high reproductive output. Furthermore, hybrids of *C. flavissima* and *C. eibli* have been filmed while spawning with both parent species in those mixed harems (Hobbs et al. 2018), indicating both acceptance in the harem and reproductive activity. This study supports the theory that, due to their high fitness and reproductive potential, hybrids are likely to persist at Christmas Island. In addition, genetic exchange and backcrossing with the parent species and other hybrids may favour a macro mutation that could allow them to survive in increasingly challenging environmental conditions (Hughes et al. 2017). A physiological study on hybrids and their parent species would be ideal to evaluate hybrids' tolerance and adaptability to increasing water temperature and decreasing pH, and further understand the potential evolutionary consequences of hybridisation.

Chapter 6. General discussion and future directions

6.1 Summary

This thesis significantly contributes towards the understanding of the causes and possible evolutionary consequences of hybridisation in coral-reef fishes, using pygmy angelfishes from the genus *Centropyge* as a model. Hybridisation happens when two genetically different species interbreed and produce viable offspring (Harrison 1993), and it can be an important process shaping the evolution of the species (Barton 2001). For example, hybrids can experience a macro mutation and be able to colonize unoccupied ecological niches (Barton 2001), or can result in the extinction of one or both of the parent species, with loss of biodiversity (Seehuasen 2006). Most of the studies on marine hybridisation have focused on genetic confirmation of the hybrids, or have simply reported the presence of hybrids and their locations (Yaakub et al. 2007; Reyes-Bonilla et al. 2012). Recent studies have highlighted the need for ecological and biological studies to better understand hybridisation in marine environment (e.g. Montanari et al 2016). However, very few studies have investigated the possible causes of hybridisation (Hobbs et al. 2013) or the biology and fitness of the hybrids compared to their parent species (Payet et al. 2016; Montanari et al. 2017), to understand the possible consequences of hybridisation.

This study is the first comprehensive study to deeply investigate the ecology (Chapter 3) and biology and fitness of the hybrids (Chapters 3 and 4), as well as their social condition (Chapter 5). With our review (Chapter 2), we reported 33 angelfish hybrids from various locations around the world, along with their abundances, distribution, and possible causes of this process, and highlighted the importance of better understanding this process in marine ecosystems. Angelfishes showed the highest proportion of hybridising species (~44%) compared to the other coral-reef fish families (Yaakub et al. 2006; Hobbs et al. 2013; Chapter 2), making them the ideal model to study hybridisation in coral-reef fishes. In Chapter 3, we tested the terrestrial theory that the lack of conspecific partners and niche overlap can facilitate hybridisation. We showed that this theory is valid in the marine environment, with the three parent species showing a high abundance disparity and similar use of macro and micro habitat, as well as food resources. Hybrids also showed the ability to spawn, and to be at least as fit as the most common parent species *C.*

flavissima in terms of growth and maturation (Chapter 4). Finally, angelfish hybrids were observed to be accepted in mixed harems with their parent species and not being chased (Chapter 5). Hybrids were more frequently the dominant male in the harems than the two rare parent species (Chapter 5). This, combined with their high level of fitness, would indicate high reproductive success, that could implicate important evolutionary consequences. This research represents a solid guideline for future studies that aim to investigate hybridisation, not only on coral-reef fishes, but also for other marine hybridising organisms.

6.2 Ecology of hybridisation and its possible causes in the marine environment

From previous studies, two main factors are believed to promote hybridisation: 1) lack of conspecific partners, and 2) resources overlap (Scribner et al. 2001; Richard and Hobbs 2015; Montanari et al. 2016). If a species is rare, an individual might be forced to interbreed with a closely-related species in the absence of conspecific partners (Hubbs 1995). Similarly, sharing the same habitat and food resources might increase the chances of encounters between individuals of different species, thus favouring hybridisation (Camp et al. 2016). Through this 14-year long study on the abundances of parent species of *Centropyge* and their hybrids at Christmas Islands, this study has shown that angelfish hybridisation has been occurring consistently throughout the years and the three parent species showed a high level of abundance disparities, thereby supporting the role of abundance disparities in facilitating hybridisation (Chapter 3). *C. flavissima* is 10 times more abundant than the two other parent species *C. eibli* and *C. vrolikii*, with all three parent species and their hybrids sharing the same macro-habitat (the reef wall). This is further supported by the proportions of the three parent species in harems. *C. flavissima* formed numerous mono-specific harems, while neither *C. eibli* nor *C. vrolikii* was found in mono-specific harems over the 14 years of this study (Chapter 5). In mixed harems, both *C. eibli* and *C. vrolikii* were found in low abundances, reflecting their rarity across the study region. This heterospecific formation of harems is likely to be promoted by all three parent species sharing the same microhabitat and food resources (Chapter 3). This high level of niche overlap is likely to enhance inter-specific encounters, allowing for the formation of mixed harem, and subsequent hybridisation. This would also explain their persistency, even if in low abundances, throughout the years (Chapter 3). It is, however,

unclear the reason behind the high abundance disparity of *C. flavissima* compared to *C. eibli* and *C. vrolikii* (Chapter 3). These three species are genetically very close (DiBattista et al. 2012), and showed very similar level of fitness in terms of growth and reproduction (Chapter 4). However, the abundance of *C. flavissima* was more than ten times higher compare to *C. eibli* and *C. vrolikii*, and this was consistent through time (Chapter 3). This could be explained by its endemism at Christmas Island (Hobbs et al. 2014), or by other factors not considered in this study. Similarly, most of butterflyfish species involved in hybridisation showed abundance disparity, with at least one of the two parent species being rare (Hobbs et al. 2013), and hybridising fish such as groupers and surgeonfishes showed habitat overlap (Frisch and van Herwerden 2006; Marie et al. 2007; Hobbs and Allen 2014; Payet et al. 2016).

Pygmy angelfishes are haremic and exhibit protogynous sex change (female to male). Harems comprise a dominant male, which is the biggest fish of the harem (TL) and spawn with each of the females at dusk (Moyer et al. 1983). Thus, growing fast, reaching maturity and changing sex are vital to be reproductive success within the harem. In this study, we showed the two rare species *C. eibli* and *C. vrolikii* were able to grow, mature and change sex as fast as the more common *C. flavissima* (Chapter 4), except for *C. eibli* that grows slightly faster but reaches a slightly smaller size (Chapter 4). The similar fitness of the three parent species may play an important role in the creation and the maintenance of mixed harems, although individuals of *C. eibli* may be at a disadvantage due to its smaller size. Having similar fitness to the common *C. flavissima*, especially in terms of growth, is likely to help the two rare parent species be accepted into mixed harems and become the dominant male. Indeed, they made up more than 60% of the mixed harems, and individuals of both species represented the dominant male and the highly ranked females (Chapter 5). Thus, regular spawning events of the dominant male with females from the other parent species could lead to hybrids. This clearly occurs, as hybrids contributed to more than 1% of the angelfish assemblage at Christmas Island during the study.

6.3 Possible consequences of hybridisation in angelfishes at Christmas Island: fitness of the hybrids and their social conditions

The fitness of an individual is vital for the continuity and evolution of the species (Barton 2001), and has usually been measured in terms of growth, susceptibility to predation and reproductive output/success (Mills and Beatty 1979; Mayr 2002). Hybrids are usually believed to be unfit compared to their parent species (Mayr 1963; Schemske 2000; Arnold and Martin 2010), but can display greater fitness than their parent species (Licht and Bogart 1989; Arnold and Martin 2010), which could eventually result in a new species evolving characteristics that allow them to inhabit new ecological niches or tolerate challenging environmental conditions (Seehausen 2004; Karrenberg et al. 2006). Here, we show that angelfish hybrids grow as fast and as big as the most abundant *C. flavissima*, as well as mature and change sex at similar sizes as their parent species. These results support the few previous studies on the fitness of reef fish hybrids, which showed that hybrids of butterflyfishes (*Chaetodon*) and groupers (*Cephalopholis*) exhibit similar growth rates to their parent species (Payet et al. 2016; Montanari et al. 2017).

Body size influences the reproductive success of terrestrial and marine species (Anderson and Fedak 1985; Balmford et al. 1992; Downing et al. 1993; Anderson et al. 2012), and in terms of haremic species, it determines the rank in haremic mating animals, which is usually associated with higher reproductive success (Moyer et al. 1983; Magnhagen and Kvarnemo 1989; Anderson et al. 2012). In this study, we show that hybrids grow to a similar size to two parent species, but a slightly larger size to the rarer *C. eibli* (Chapter 4). Also, the bigger size of the fish corresponds with the higher ranks in the harem (Chapter 5). For haremic angelfishes, body size is important for females as well. When the male is removed from the harem, it is the biggest female that changes sex and becomes the dominant male 'taking over' the harem (Shapiro 1979; Moyer et al. 1983; Sakai 1997). Thus, hybrids can become the dominant male and reach high ranks among the females, indicating that they are able to enter or form harems and persist in those harems.

In addition to body size, the size of the harem is an important factor that influences the reproductive output of a species in terrestrial and marine environments (Hoffman 1985; Bercovitch 1986; Cowlishaw and Dunbar 1991; Modig 1996; Ang and Manica 2010;

Wong 2011). For haremic fish, more females in a harem will enhance chances of spawning with a male and passing on their genes to future generations. Here, we show no significant difference in harem size between mixed harems with hybrids, mixed harems without hybrids, and pure harems of the most abundant species *C. flavissima* (Chapter 5). However, the percentage of hybrid males in mixed harems is actually higher than the two rare parent species *C. eibli* and *C. vrolikii* (Chapter 5). The combination of hybrid angelfishes displaying similar growth and size, being in similar sized harems, and reaching similar ranks as both males and females of parent species in mixed harems, indicates that their reproductive output would be similar to all three parent species in those mixed harems, but lower than *C. flavissima* due to this species forming large numbers of mono-specific harems.

Since hybrids exhibited fully developed gonads and the ability to spawn through the presence of postovulatory follicles (Chapter 4) and observed spawning behaviour (Hobbs et al. 2018), it is highly plausible that they can produce recruits. This is supported by the observation of likely hybrids crossed with parent species in the study region (Appendices 4.1 and 4.2), and by molecular evidence of backcrossing and introgression between parent species (DiBattista et al. 2016b), suggesting the capacity of hybrids to breed back with their parent species and other hybrids. Regardless, their relatively high reproductive potential helps to explain their long-term persistence at Christmas Island, even if they are present in low abundances, but in similar numbers to the two rarer parent species (Chapter 3). The similar level of fitness of hybrids to their parent species, allowing them to enter or form and persist in harems, provides them with the capacity to transmit their genes to future generations (Barton 2001; Arnold and Martin 2010). Through introgression and high genetic recombination, hybrids could have the potential to inhabit new niches or adapt to survive challenging environmental conditions (e.g. increasing water temperature and decreasing pH) (Barton 2001; Seehausen 2004; Mallet 2007; Arnold and Martin 2010). For example, fish hybrids from the genus *Poeciliopsis* showed adaptation to colder water temperature (Bulger and Schultz 1982), and butterfly hybrids from genus *Lycaeides* inhabited high-elevation alpine habitats unoccupied by either parent species (Gompert et al. 2006). Concluding, this multi-scale study is the first of a kind, investigating ecological and biological aspects of hybridisation in the marine environment, and represents a significant advance in the literature on the possible causes and consequences of hybridisation in coral-reef fishes.

6.4 Future directions

The high number of angelfish hybrids (Chapter 2), their mating systems, and their world-wide distribution, make this family a perfect model family to study hybridisation. With this study, we showed that angelfish hybrids have the fitness and the social condition to spawn, however, we could not test their viability. To achieve that, a multi approach study is needed: in vitro fertilisation experiments could be combined with genetic analysis to determine whether the hybrids are reproductively viable and potentially play a role in evolutionary processes, or whether they provide an evolutionary "dead-end".

Here, we demonstrated that hybrids are socially accepted by their parent species, and that hybrids form harems with the three parent species according to their relative abundances (Chapter 5). The next step could be to determine whether there is preference for particular parent species or hybrids for spawning. Mate choice experiments in aquaria could be undertaken to understand mating preferences of the hybrids, and determine the physiological processes (olfactory, sight, or sound) that play a role in partner choice. These types of experiments have been carried out before, and showed that body colouration influenced mate choice in other fish families such as Cichlidae (Seehausen and van Alphen 1997) and Gobiidae (Amundsen and Forsgren 2001). Such an approach may help to explain the distinct disparity that occurs in the abundances of the parent species (Chapter 3) despite those species exhibiting similar fitness (Chapter 4) and ability to reach high ranks in mixed harems (Chapter 5).

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Appendices

Appendix 3.1 – Angelfish parent species (left and middle) and the three possible hybrids (right). A) *Centropyge flavissima* + *C. eibli*; B) *C. vrolikii* + *C. flavissima*; and C) *C. eibli* + *C. vrolikii*. The three angelfish species had distinct body colours and patterns, and the resulting hybrids displayed a mix of these colours. Photos by Tane Sinclair-Taylor.
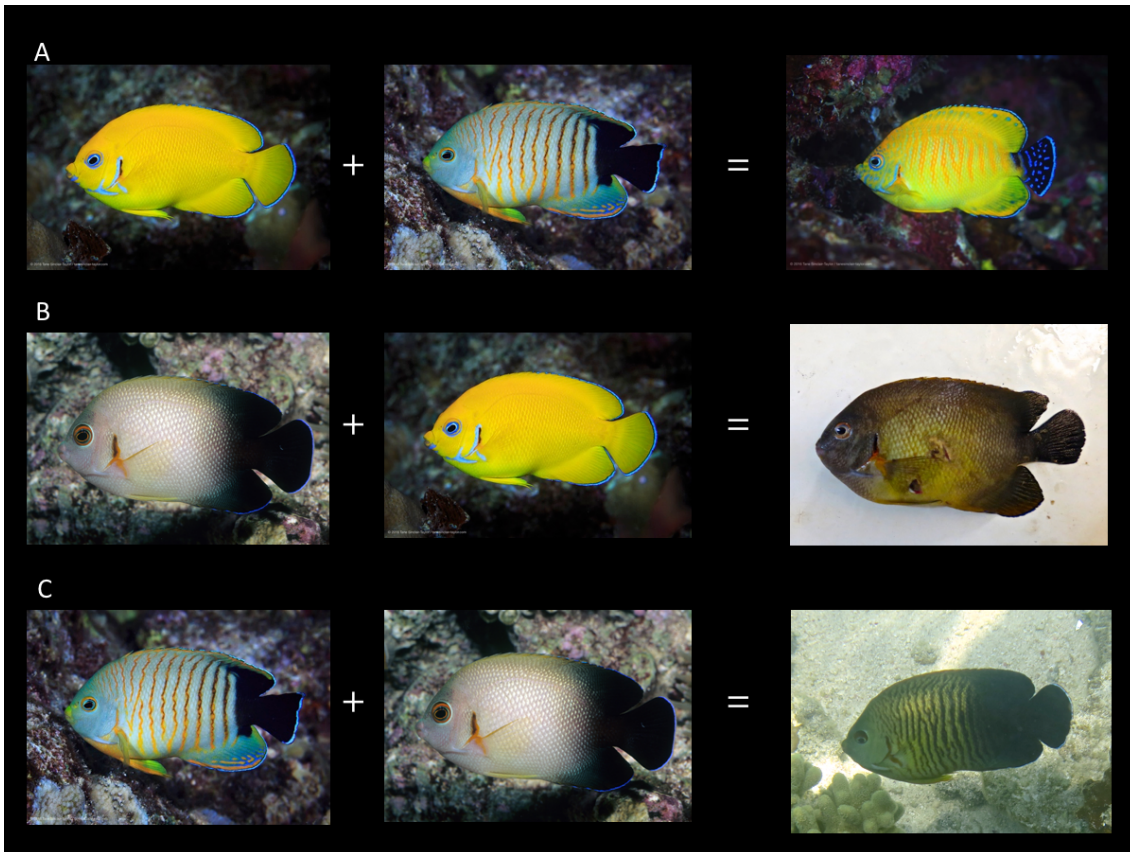
Appendix 4.1 – Putative F1 hybrid backcrossed with its parent species. Possibly a *Centropyge flavissima* × *C. eibli* F1 hybrid crossed back with *C. flavissima*. The bright yellow body colour is typical of the parent species *C. flavissima*, however, faded orange transversal stripes on the body are still visible, typical of *C. eibli*. Photo by Tane Sinclair-Taylor.

Appendix 4.2 – Putative tribrid caught at Christmas Island. This individual might be a hybrid (any of the three) backcrossed with one of its parent species. The individual exhibited body colours and patterns from each of the three parent species: the yellow body colour from *Centropyge flavissima*, the black shading of *C. vrolikii*, and some stripy pattern towards its tail mixed with an orange and blue pattern on its anal fin, typical from *C. eibli*.