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Finding Nemo's place in a complex animal society: An exploration of the
behavioural and hormonal correlates of dominance in *Amphiprion ocellaris*

by

Eugene Cheung

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An exploration of the behavioural and hormonal correlates of dominance in
Amphiprion ocellaris

by

Eugene Cheung

APPROVED BY:

_____
Dr. Christina Semeniuk, External Reader
Great Lakes Institute for Environmental Research

_____
Dr. Oliver Love, Internal Reader
Department of Biological Sciences

_____
Dr. Dennis Higgs, Advisor
Department of Biological Sciences

July 30, 2015

Declaration of Co-Authorship

I hereby declare that this thesis incorporates material that is result of joint research, as follows:

This thesis incorporates the outcome of a joint research endeavour undertaken in collaboration with Dr. Oliver Love, Dr. Dennis Higgs, and Pauline Capelle. This collaboration is covered in Chapter 3. In all cases, primary contributions, experimental designs, and data analysis and interpretation were performed by the primary author. The contribution of co-authors was based in the provision of advice on experimental design and performance of experimental protocols.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from each of the co-authors to include the above materials in my thesis.

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Abstract

Exploration of the mechanisms underlying conflict resolution has been key to our understanding of the dynamics driving the formation and organization of complex animal societies. This thesis examines the role of aggression and individual variation on dominance hierarchies and the correlates of expression of cortisol, 11-ketotestosterone, and testosterone on individual social status in novel size-matched *Amphiprion ocellaris* dominance hierarchies. Here, I report that greater aggressiveness relays higher dominance status during hierarchy establishment, as well as during experimental recruitment of highly aggressive smaller individuals into established groups. Additionally, I show that cortisol expression profiles are related to social status in both unstable and stable hierarchies, with top-ranked dominants and lowest-ranked subordinates demonstrating stress of dominance and subordination respectively. These results offer a contrasting elucidation to the size-based hierarchy hypothesis typically implicated in modulating anemonefish social structures and provide evidence indicating that dominance may be driven by variation in individual aggressiveness and stress profiles.

Dedication

To the memory of my grandfather.

Acknowledgments

To my best friend and the love of my life, Mitchell Elliott, I thank you for your love and support. Words cannot express my gratitude for the support and encouragement you've given to me throughout this endeavour.

To the wonderful graduate student community in Biology, I am grateful for the times and memories we shared together. Long live the CPC. Partington Palace will stand forever.

To my parents, I thank you for your sacrifice in leaving our native home and bringing me to Canada where I have been able to pursue a deeply enriching education.

And finally, to my cats, Pepper, Pico, and Potato, meow meow meow meow.

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CHAPTER I: GENERAL INTRODUCTION

In animal societies, cooperation is defined as collective behaviour that benefits the fitness of both the recipient and donor (Trivers 1971; Axelrod and Hamilton 1981; Clutton-Brock 2009). The multitude of breeding systems that exist across taxa have lent themselves to the increasing focus and study of cooperative reproductive aggregations (e.g., Dugatkin and Mesterton-Gibbons 1996; Taborsky 2001; Krams *et al*. 2008; Schino and Aureli 2009). However, inconsistent application of terminology pertinent to cooperative reproduction has presented a vital need to reassess whether certain taxa are actually engaging in cooperative behaviours that benefit the immediate fitness of breeders with potential fitness benefits for non-breeders rather than non-cooperative behaviours typically associated with living in social aggregations.

Cooperative reproduction presents an evolutionary paradox through its proclivity in immediate unidirectional benefit to the reproductive output and fitness of recipients, with potential fitness benefits for donors. As defined by Darwinian criteria, natural selection and evolution should favour traits and behaviours that benefit the biological fitness of the individual that retains them (Darwin 1859). However, altruistic behaviours associated with cooperative reproduction act in direct contradiction to this mandate via the enhancement of one individual's reproductive output, while omitting that of another (Hamilton 1963; Matessi and Jayakar 1976). A variety of models have been proposed as viable explanations to behaviours associated with cooperative reproduction (e.g.,

Von Neumann and Morgenstern 1945; Trivers 1971; Clutton-Brock 2009), all of which provide consistent criteria to define qualities of cooperatively reproducing species.

Across nearly 30,000 species, fishes display an abundance of reproductive strategies and mating systems. Surprisingly, cooperative breeding has not been comprehensively explored through experimental means beyond singular examples all found within the gonochoristic family Cichlidae (e.g., Taborsky 1984; Taborsky 1985; Brouwer *et al*. 2005; Heg *et al*. 2005). Gregarious sequential hermaphroditic marine fishes present a unique opportunity to engage the topic of cooperation and cooperative breeding due to the rigidity and particular organization of their social and reproductive hierarchies (Fricke and Fricke 1977; Fricke 1979; Warner 1988; Godwin 1994). While some species have been suggested to display cooperative behaviours (e.g., Buston 2003ab; Wong *et al*. 2007; Pinto *et al*. 2011), the validity of these assertions requires deliberation in the context of themes that underlie the current understanding of cooperation.

The primary objective of this paper is to review mating systems in species from three families of hermaphroditic marine fishes and to assess whether or not these mating systems qualify as cooperative breeding arrangements. I begin by briefly exploring our contemporary understanding of cooperation and cooperative breeding, with a particular focus on the well-studied *Neolamprologus pulcher* (see review by Wong and Balshine 2011) as a model of cooperative reproduction in fishes. Then, I briefly examine possible examples of cooperation in particular

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species within the following families: Pomacentridae - *Amphiprion percula,*
A.polymnus, A.melanopus; Pomacanthidae - *Holacanthus tricolor, Centropyge argi*;
and Labridae - *Labroides dimidiatus, Thalassoma bifasciatum.* Finally, I evaluate
the legitimacy of describing these species as cooperative breeders under current
criteria of cooperation and suggest avenues of further study.

Theory

      Expanding research on cooperation across taxa has coincided with the
establishment of a confusing array of interchangeable terminology. To retain
consistency throughout this paper, I define "cooperative behaviour" in the context
of cooperative reproduction as a binary interaction that presents immediate
benefits to the reproductive output and fitness of the recipient with potential to
incur benefits to the donor. Collectively, I define "cooperation" as the process in
which cooperative behaviour between individuals invokes mutual benefits.

      Cooperative behaviour retains a fundamental contradiction to natural
selection and evolutionary theory that dictates individual efforts should be
directed towards maximizing individual survivorship and reproductive output.
Cooperative behaviour in reproductive contexts incorporates properties of
alloparental behaviour typically associated with the amelioration of one
individual's (recipient) fitness and reproductive output, while omitting that of
another (donor) (Hamilton 1963; Trivers 1971; Smith 1998). This definition is
inherently paradoxical in the context of the expectation that individuals should

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invest in propelling their own fitness. However, the conventional hypotheses proposed for understanding cooperation outline benefits for both recipients and donors, which in turn, indicate the function of reciprocal mutualism between recipients and donors.

A commonly accepted paradigm for understanding the function and evolution of cooperation in animal societies is the kin selection hypothesis (Smith 1964; Eberhard 1975; Clutton-Brock 2002; Foster *et al*. 2006). There are various examples of complex social communities that are divided into reproductive and non-reproductive phenotypes. Eusocial insects (e.g., Hymenoptera, Coleoptera, Isoptera) provide a prominent example of obligate cooperation that likely relies on kin selection due to the coexistence of sterile and reproductive castes (e.g., Ross and Keller 1995; Keller and Chapuisat 1999; Ratnieks *et al*. 2006). Kin selection provides a viable explanation for the continued subsistence of sterile workers in eusocial insect communities (Eberhard 1975; Queller and Strassmann 1998; Clutton-Brock 2002; Foster *et al*. 2006). In the absence of reproductive capability, sterile workers must rely on alternate means of maintaining propensity towards fitness (Queller and Strassmann 1998). Here, cooperative behaviour (e.g., territory defense, anti-predator behaviour, foraging) allows for sterile castes to contribute to the fitness of kin by protecting both individual and collective survivorship (direct benefit to reproductive recipients), while also improving their own fitness by proxy (cryptic benefit to sterile donors) due to high relatedness

with reproductive individuals (Queller and Strassmann 1998; Clutton-Brock 2002).

The notion that relatedness and kin selection provide a sufficient mechanistic elucidation of the evolution and function of cooperative societies has been challenged by Clutton-Brock (2002, 2009) for several reasons: (1) whether they are cooperative or not, most permanent aggregations of social animals are typically composed of related individuals; (2) the value of indirect benefits incurred by cooperation may be grossly overestimated due to the costs associated with group living; and (3) the cost of non-breeding individuals helping within their social communities may be overestimated since the substantial energetic costs associated with cooperation are typically proportional to individual capacity to contribute. While it is likely that kin selection enables the continued evolution and function of cooperative societies, exemplified by advanced eusocial communities, one can posit that the existence of cooperation is underscored more heavily by quintessential benefits borne of group living.

To assert that kin selection is a sufficient general explanation for the occurrence of complex cooperative animal societies would then invoke the question as to why all permanent aggregations of social animals, which are typically composed of relatives, do not necessarily function cooperatively. This dilemma is then distilled to the fundamental appliance of group living, which underlies all cooperative societies. Group living confers a variety of benefits, including territory defense, predator threat dilution, facilitated foraging, and

mating opportunities (Rubenstein 1978). Aggregations also incur costs, such as within-group competition, increased pathogen transmission, and conspicuousness to predator threats (Rubenstein 1978; Whitehouse and Lubin 2005; Majolo *et al.* 2008). Hence, the formation of social groups stands on an individual basis of consideration as to whether the benefits of group living outweigh the overall costs (Rubenstein 1978; Clutton-Brock 2002). The existence of social groups entails the implicit assumption that the benefits of forming an aggregation outweigh that of solitary life (Rubenstein 1978). Hence, group living can be attributed to the overall benefits incurred via participation in social groups and provides a possible explanation to social living in addendum to kin selection in cooperative societies.

Within non-cooperatively breeding animal societies that also display clearly defined and enforced reproductive and non-reproductive social roles, the benefit of remaining in such an aggregation particularly as a non-reproductive individual is not immediately clear. Fundamentally, participation in group living implies that the benefits of remaining in a social group largely outweigh the costs. In a cooperatively breeding society, the forfeiture of individual fitness by helpers is rectified via group living benefits (Rubenstein 1978; Seeley and Visscher 1988) and indirect fitness benefits through the reproduction of kin (Clutton-Brock 2002; Clutton-Brock 2009). In non-cooperatively breeding societies, it is likely that remaining in a community with a strict breeding hierarchy incurs benefits to survivorship (with the potential to attain reproductive status barring high level changes within the community) at the concurrent decline of reproductive output

(Wong *et al*. 2007; Cant 2011). These benefits are ostensibly moderated by mechanisms underlying the function of sociality as a whole: (1) reciprocal altruism enforces a tit-for-tat scheme, in which contributing to another's survivorship or reproductive output can translate into eventual reciprocation of the same benefits (Trivers 1971); (2) the prisoner's dilemma asserts that cooperation can confer great benefit at lower risk than individual action (Axelrod 1980; Clements and Stephens 1995; Clutton-Brock 2009); and (3) willful divergence from mutualistic and reciprocal behaviour in favour of selfish gains can be accounted for by the threat of punishment (e.g., subordination via agonistic attacks, withholding of resources, eviction from group) within the social community (Clutton-Brock and Parker 1995; Buston 2003ab; Wong *et al*. 2007; Ang and Manica 2010ab). Overall, the coordination of social behaviour and group living may be sustained by the properties of reciprocal mutualisms, but qualifying behaviour and complex communities as "cooperative" may require further consideration beyond these mechanisms.

Cooperation in *Neolamprologus pulcher*

With a vast array of complex social behaviours, cichlids have been the focal model organisms in a multitude of behavioural studies (see review by Rossiter 1995). Cooperative behaviour and breeding systems have been investigated heavily in the Tanganyikan cichlid *Neolamprologus pulcher* (see review by Wong and Balshine 2011). This species is typically found in colonies ranging from 2 to

30 individuals per colony (Balshine *et al*. 2001). These colonies are composed of a single socially dominant breeding pair and smaller subordinate helpers (Balshine *et al*. 2001; Heg *et al*. 2005). Breeding pairs are not always monogamous, with some males exhibiting haremic polygyny in which each harem is composed of its own dominant female and subordinate helpers (Desjardins *et al*. 2008; Taves *et al*. 2009). All individuals within a colony engage in territory defense, predator defense, and rearing of offspring (Balshine *et al*. 2001; Schurch and Heg 2010). However, breeding females and helpers commit a heavier investment to colony maintenance tasks (Balshine *et al*. 2001; Desjardins *et al*. 2008).

Relatedness within colonies has recently become the subject of inquiry due to the implied necessity for high relatedness between individuals to persist in order for kin selection to function (Dierkes *et al*. 2005; Stiver *et al*. 2005). It has been found that within individual colonies, approximately 85% of subordinate helpers are not offspring of the dominant breeding pair (Stiver *et al*. 2005). However, younger subordinates are typically more related to the breeding pair than older subordinates (Stiver *et al*. 2005). This degree of relatedness is higher when comparing subordinate helpers to the breeding female as opposed to the breeding male (Dierkes *et al*. 2005; Stiver *et al*. 2005).

Cooperation (or "helping") in non-breeding *N. pulcher* has been defined to incorporate behaviours including: (1) egg/larvae cleaning and defense, (2) territory cleaning, and (3) territory defense from competing conspecific colonies and heterospecific competitors (Taborsky 1984; Balshine *et al*. 2001). Within

subordinate helper social hierarchies, the degree of helping behaviour is relative to individual social ranking, sex, and relatedness to the breeding pair (Stiver *et al*. 2005). Studies of *N. pulcher* colonies in closed laboratory settings have provided evidence suggesting that helper behaviour results in increased offspring survivorship and territory retention (Bergmuller and Taborsky 2005). Multiple hypotheses have been suggested as viable mechanisms as to why subordinate *N. pulcher* helpers sacrifice individual fitness and reproductive output: (1) indirect fitness benefits are incurred via helping related breeding individuals (Hamilton 1964); (2) by staying in a colony and helping, a helper can potentially gain access to a breeding female via sneaky breeding tactics (Fitzpatrick *et al*. 2006); (3) helping behaviour may be considered as a form of "payment" required to remain within the colony (refer to earlier description of threat of punishment within social groups) (Gaston 1978; Stiver *et al*. 2005); (4) enhancement of group size via increased breeder offspring survival can contribute to increased helper survival and potentially impact eventual helper reproductive output (Balshine *et al*. 2001); and (5) helping might be considered a qualifier of social prestige that can result in increases in social status (Zahavi 1995). Recently, several of these hypotheses have been explored in the context of existent evidence pertaining to the social behaviour of *N. pulcher* (see review by Wong and Balshine 2011).

1. Kin selection

Within cooperative societies, kin selection is expected to be a primary driver in the evolution of helping behaviour under the critical assumption that

individuals within cooperative aggregations are related (Smith 1964; Eberhard 1975; Clutton-Brock 2002; Foster *et al*. 2006). Through helping, breeders and helpers are able to benefit through the function of inclusive fitness (Hamilton 1984). Several key predictions that underscore the role of kin selection in driving helping behaviour in *N. pulcher* have been suggested:

i.   Kin recognition

If relatedness is an assumed driver of helping behaviour, then a key prerequisite would be a helper's capacity and ability to distinguish kin from non-kin. Previous reports have provided evidence indicating *N. pulcher* ability in identifying familiar individuals when given a binary choice between familiar and unfamiliar conspecifics (Jordan *et al*. 2010). However, the ability to discriminate between the differing degrees of familiarity has been recorded across both cooperative and non-cooperative fish species (e.g., Arnold 2000; Gerlach *et al*. 2008; Jordan *et al*. 2010). In addition, helper preference for familiar conspecifics may not be indicative of genetic relatedness. More recently, evidence has been provided for helper preference of unfamiliar kin over unfamiliar non-kin (Le Vin *et al*. 2010). Hence, capacity to discriminate between kin and non-kin, as well as preference for kin rather than familiar conspecifics, may suggest that kin recognition plays a critical role in helping.

ii.   Helpers preferentially help kin

Experimental manipulations of *N. pulcher* groups have been performed to assess the amount of help that helpers contribute when placed with either related

or unrelated breeding pairs (Stiver *et al.* 2005). However, the experimental design has been suggested to be confounded by the fact that helpers were placed with breeding pairs in which they may have been related to either the male or female but not both (Wong and Balshine 2011). Additionally, this study yielded contradictory results suggesting that helpers contribute more help to non-kin rather than kin (Stiver *et al*. 2005). Recommendations to considering the probability of helping rather than absolute values of contribution (Emlen and Wrege 1988) have been considered in avian models, but require validation and use in *N. pulcher* studies in order to elucidate the contribution of kin selection in helping behaviour particular to this species.

2. Payment

Helping behaviour has been suggested as a form of payment that helpers contribute to breeders in order to maintain a position within helper dominance hierarchies, as well as the social group as a whole (Gaston 1978; Stiver *et al*. 2005). The enforcement of "rent payment" occurs via the threat of punishment by social dominants (Clutton-Brock and Parker 1995; Buston 2003a; Wong *et al*. 2007; Ang and Manica 2010ab). While this mechanism is unlikely to have contributed as the sole driver underlying the evolution of helping behaviour, the costs incurred by retaining both breeders and non-breeders within a social group beyond assistance in brood-rearing and amelioration of breeder reproductive output may provide substance to the overall necessity for consistent helper contribution in order to maintain a place in the group.

i.   Punishment

Previous reports have provided evidence for punishment in the occurrence

of reduced help from helpers. In one such investigation, large helpers were

removed from groups to prohibit helping behaviour (Balshine-Earn *et al*. 1998).

Upon being returned to the group, the focal helpers were subject to more

aggressive behaviour from other similarly sized helpers. While this may suggest

the occurrence helper punishment by helpers, a follow-up study indicated that the

increased aggression towards the removed helpers may have actually been a

result of size hierarchy-based social conflict rather than actual punishment (Field

and Cant 2009). Further experimental manipulations of *N. pulcher* groups that

can demonstrate payment and punishment as a critical driver of helping

behaviour will be needed in order to support the hypothesis that the evolution

and maintenance of help was based in this complex social mechanism.

3. Prestige

Social "prestige" has been suggested to be a beneficiary of helping

behaviour (Zahavi 1995). In the context of cooperation, the overall contribution

of helping behaviour can be considered an honest signal of individual quality

(Zahavi 1995; Gintis *et al*. 2001). Individuals that help more can attain higher

social prestige, and consequently, higher social status (Gintis *et al*. 2001). The

acquisition of higher social status can translate into potential mating opportunities

for helpers, as well as collaborative assistance from other helpers (Zahavi 1995;

Gintis *et al*. 2001). While it has not been explored heavily in *N. pulcher,* the notion

of social prestige contributing to the evolution and maintenance of helper behaviour will require further investigation in order to consider the complex dynamics underlying signaling of social status and dominance relationships.

These three hypotheses have received partial support through experimental evaluations of their applicability to the *N. pulcher* system. Further investigation of the critical mechanisms driving cooperation and helping behaviour will be necessary to provide a more comprehensive understanding of the cooperative social dynamic in this model. Nonetheless, the foundation established by investigations of *N. pulcher* provides potential applicability and translational relevance to evaluation of cooperation in other gregarious fish species.

Cooperation in gregarious sequential hermaphroditic marine fishes

The mechanisms underlying the function of social communities in gregarious sequential hermaphroditic marine fishes have been the subject of continued study due to distinctions from social behaviour typically expected in gonochoristic fishes (e.g., Warner 1988; Godwin 1994; Buston 2003ab). Many of these studies have asserted that haremic hermaphroditic fish communities exhibit cooperative breeding behaviour (e.g., Buston 2003ab; Ang and Manica 2010ab; Pinto *et al*. 2011). However, the validity of claiming that a social community incorporates cooperation into its function and survival requires further corroboration under specific qualifiers. In Table 1.1, I characterize several gregarious hermaphroditic marine fishes according to the criteria used earlier to

describe cooperation in *N. pulcher*. These criteria include (1) within-group relatedness, (2) brood rearing (breeder offspring cleaning and defense by non-breeders), and (3) territory maintenance and defense by non-breeders. None of the seven species considered were compliant with criteria established by the *N. pulcher* model. In the absence of evidence indicating helper behaviour, particularly in brood rearing, the description of any of these species as cooperative breeders would largely be a misnomer. While within-group relatedness, a necessary prerequisite for the kin selection hypothesis, was typically reported to be moderate, many studies have indicated that permanent animal aggregations are generally composed of related individuals regardless of whether cooperation occurs or not. Nonetheless, further consideration should be given to the invocation of individual behavioural complexity that contributes to the formation of hermaphroditic marine fish aggregations.

A prime and puzzling example of this inexact designation comes from recent studies of the anemonefish *Amphiprion percula*. *A. percula* has been persistently described as a cooperative species (e.g., Buston 2003ab; Buston 2004; Buston *et al*. 2007). However, in all descriptions of the species, there are no existent records of explicitly cooperative behaviour (e.g., helping behaviour from subordinate non-breeders). Additionally, cooperation in animal societies is typically expected to coincide with overall fitness and survivorship benefits for both breeders and non-breeders (Trivers 1971; Axelrod and Hamilton 1981; Clutton-Brock 2009). In a study that conducted experimental removals of non-

breeding subordinates (Buston, 2004), five hypotheses were tested pertaining to the impact of this community alternation to an established *A. percula* colony: (H1) survival benefit, (H2) growth benefit, (H3) reproductive benefit, (H4) eviction cost, and (H5) mate replacement benefit. Across the five hypotheses, it was found that there were neither positive nor negative effects of the presence of non-breeders (H4) on breeder survival (H1), growth (H2), and reproduction (H3). The only negligible benefit that non-breeder presence incurred on breeders was the service of mate replacement in the instance of breeder widowing (H5). In combination of the dearth of appreciable fitness benefits incurred by manipulation of social structures, as well as no recorded instances of helping behaviour, the immediate deduction drawn is that *A. percula* communities do not demonstrate cooperative behaviour. The only distinct similarity to cooperative societies would be the division of reproductive and non-reproductive social castes. While many hermaphroditic marine fishes occur in aggregations with strictly defined reproductive hierarchies (e.g., breeder and non-breeders), this should not serve as an immediate denotation of cooperation within a permanent social aggregation. Consideration of criteria established in the investigation of systems explicitly known to exhibit cooperation and cooperative behaviour should be applied when assessing species that may have similar characteristics to models like *N. pulcher*.

Synthesis and future directions

Complex animal societies depend on a variety of integrative mechanisms to persist and in turn, yield fitness benefits to individuals whom contribute to the continued survival of the group (Clutton-Brock 2002; Clutton-Brock 2009). However, cooperation and cooperative behaviour should be better differentiated from behaviour typically associated with group living regardless of the degree of sociality (e.g., territory defense, predator threat dilution, facilitated foraging, more mating opportunities). Particular consideration should be given to animal aggregations that have stark divisions between dominant breeders and subordinate non-breeders, in which there may be a superficial similarity to well-defined cooperative species, but an absence of actual cooperation in which subordinate non-breeders commit to an altruistic cessation of individual reproductive output in order to improve that of dominant breeders. Hence, I recommend the following criteria to assess whether cooperation is occurring in an animal aggregation: (1) offspring rearing (egg/larvae/fry cleaning and defense) by non-breeders, (2) territory defense by non-breeders, and (3) significant benefits to the survival, growth, and reproductive output of breeders by the presence and contribution of non-breeders. Collective action with the purpose of sustaining a social community is likely the conclusive effect of individual behaviour committed to maximizing individual fitness and in effect, the collective fitness of the community itself (Clutton-Brock 2009). To further understand the overall function of social groups and why some species choose to be gregarious while others do not, consideration should be given to the mechanistic

underpinnings of how animal societies form and the impact of individual contribution to the functioning of social groups.

Thesis objectives

This thesis is comprised of two primary objectives. First, I assessed the role of individual behavioural variation in determining social dominance in novel size-matched groups of the false percula anemonefish *Amphiprion ocellaris*. This objective evaluated the legitimacy of the size-based hierarchy hypothesis (Buston 2003b) and considered the impact of individual aggressiveness on dominance rank. Second, I assessed the relationship between *A. ocellaris* dominance status and individual variation in waterborne hormone expression profiles, with specific consideration to cortisol, 11-ketotestosterone, and testosterone. By classifying and describing the fine-point mechanisms that modulate individual dominance status and behaviour in social aggregations, this thesis contributes to the elucidation of the underlying drivers behind the organization of complex animal societies.

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Table 1.1: Species appropriation to parameters that define cooperation in *N. pulcher*

Species	Within-group relatedness	Offspring cleaning and defense by non-breeders	Territory maintenance and defense by non-breeders	References
Labridae (protogynous)				
Labroides dimidiatus	Low to moderate; frequent between-group subordinate movement	Free-spawning, offspring care does not occur	Yes	Kuwamura 1984; Nakashima *et al.* 2000
Thalassoma bifasciatum	Moderate	Free-spawning, offspring care does not occur	No, territory defense typically performed by dominant males	Kuwamura 1984; Warner 1988,
Pomacentridae (protandrous)				
Amphiprion percula	Moderate	No, primary parental care only	Yes	Buston 2004
Amphiprion melanopus	Moderate	No, primary parental care only	Yes, territory defense almost exclusively performed by subordinate non-breeders	Godwin 1994
Amphiprion polymnus	Moderate	No, evidence suggesting limited primary parental care prior to pelagic dispersal	Yes	Jones *et al.* 2005; Saenz-Agudelo *et al.* 2014
Pomacanthidae (protogynous)				
Holacanthus tricolor	Moderate	Free-spawning offspring care does not occur	Moderate, high between-group movement of subordinate females	Hourigan and Kelley 1985
Centropyge argi	Moderate	Free-spawning, offspring care does not occur	Yes	Moyer *et al.* 1983

CHAPTER II: ATTACK OF THE CLOWNS – AGGRESSION AND DOMINANCE IN THE FALSE PERCULA ANEMONEFISH *AMPHIPRION OCELLARIS*

Introduction

Dominance hierarchies serve to allocate limited resources preferentially to socially dominant individuals while avoiding costly competitive agonistic encounters between members of the same social group (Alexander 1974; Alexander and Borgia 1978; Ellis 1995; Rubenstein and Kealey 2010). In cooperatively-breeding taxa, dominance confers reproduction to one or several individuals (Alexander 1974; Ellis 1995; Nakano 1995; Pusey *et al.* 1997; Monnin and Peeters 1999). Hierarchies are typically formed based on ranks established from competitive dynamics (Alexander 1974; Drews 1993; Ellis 1995; Rubenstein and Kealey 2010) or asymmetries in size (Chase 1982; Koebele 1985; Buston and Cant 2006) or age (Chase 1982; Hughes and Strassmann 1988; Tsuji and Tsuji 2005). Depending on the conventional measure of dominance in a particular species, individuals may attain preferential access to resources or breeding status through such asymmetries (Chase 1982; Buston and Cant 2006; Rubenstein and Kealey 2010), but the mechanisms driving hierarchy establishment and maintenance remain unclear.

Size-based asymmetry (Rubenstein 1981; Chase 1982; Koebele 1985; Buston 2006) and individual variation in aggressiveness (Alexander 1974; Drews 1993; Ellis 1995) have been argued to be common drivers of dominance status and hierarchy formation. Size hierarchies occur in concert with social hierarchies within many species (e.g. Forrester 1991; Koebele 1985; Buston and Cant 2006)

and may indicate the presence of competition between members of the same social aggregation (Heg *et al.* 2004; Buston and Cant 2006). The occurrence of size hierarchies that incur adaptive benefits through concurrence and regulation of social hierarchies have been documented in cooperatively breeding hermaphroditic marine fishes including gobies (*Gobiodon* spp. and *Paragobiodon* spp.: Nakashima *et al*. 1996; Kuwamura and Nakashima 1998; Munday 2002) and angelfishes (Pomacanthidae: Moyer and Zaiser 1984; Thresher and Brothers 1985; Aldenhoven 1986; Sakai and Kohda 1997). The regulation of size and social hierarchies in these species has been suggested to occur via competitive asymmetries in the form of resource holding capacity and aggression (Fricke and Fricke 1977; Fricke 1979; Aldenhoven 1986; Munday 2002; Rubenstein and Kealey 2010), in which dominants direct aggressive and competitive behaviors associated with retaining resources towards subordinates. Previous studies have largely focused on consistent step-wise variations in size in pre-existing groups of hermaphroditic marine fishes that also form cooperatively breeding linear hierarchies, while also providing evidence for a direct correlation between size, dominance and breeding status (Fricke and Fricke 1977; Forrester 1991; Buston 2003; Heg *et al*. 2004). However, little attention has been directed at mechanistic drivers of dominance and size differences aside from the service of minimizing intraspecific conflict and hypotheses on risk-taking through assessment of aggressive risk via visual cues.

An ideal model for the examination of size-based dominance hierarchies is the anemonefish (*Amphiprion ocellaris*), a protandrous species that lives in haremic aggregations in sea anemones along coral reefs (Moyer and Nakazono 1978; Fautin and Allen 1992; Iwata *et al*. 2008). Each aggregation maintains a linear dominance hierarchy that consists of a dominant breeding pair and subordinate non-breeding males and represents a queue for dominance and breeding status (Fricke 1979; Buston 2003). Rank changes in established groups occur only on the removal of higher ranked individuals (Hattori 1991; Buston 2003). If the top-ranked breeding female is removed, the breeding male undergoes protandrous sex change to assume the vacated position as breeding female, while the highest-ranked non-breeder becomes the breeding male (Fricke and Fricke 1977; Buston 2003). In studies of wild populations, it has been suggested that body size is a direct correlate of social dominance across the *Amphiprion* and *Premnas* genera (Buston 2003). However, it is unknown as to whether the mechanism underlying dominance is itself driven by size or whether dominance achieved through alternative competitive asymmetries drives growth and size. The purpose of the current study is to examine the roles of individual variation in size, competitive behaviors, and behavioral boldness in the establishment and maintenance of dominance hierarchies in the false percula anenomefish *Amphiprion ocellaris*.

Materials and Methods

Subjects and holding conditions

Captive-bred 5-month-old juvenile male false percula anemonefish (*Amphiprion ocellaris*) bred from an Indonesian wildstock were obtained from a regional supplier (SeaUMarine, Ontario, Canada). Size-matched individuals (total length ranged between 32-41 mm and arranged in groups according to size within this range) were housed in 76 liter glass aquaria (length x width x depth: 77 cm x 33 cm x 33 cm) in groups of 5 in the animal housing facilities at the University of Windsor (Ontario, Canada). Housing aquaria were filled with dechlorinated tap water supplemented with 30 g/L aquarium salt (Instant Ocean – United Pet Group, Virginia, USA). External power filters (Marineland – United Pet Group, Virginia, USA) provided continuous three-stage filtration. Thermostat-controlled aquarium heaters (EHEIM GmbH and Co KG, Deizisau, Germany) held water temperature at 26°C. A 12L:12D light cycle was maintained with lights on at 0630h. Each aquarium included a large rock, replica anemone, and 3 cm deep aragonite sand substrate. The fish were fed twice daily with a pulverized mixture of dry food composed of Tetramin flake (Tetra Corp., Melle, Germany) and dried brine shrimp (Hikari Fish Industry Group, Yamasaki, Japan). Diet was supplemented with a weekly feeding of garlic-soaked defrosted brine shrimp cubes (*Artemia salina*, Hikari Fish Industry Group, Yamasaki, Japan).

In total nine (n=9) experimental groups and thirty-three individual fish (n=33) were tested and included in analysis. All experiments were performed for two

separate cohorts (Coh 1, group n=4, fish n=19; Coh 2, group n=5, fish n=14) to test repeatability of hierarchy establishment (experiment 1) and to test effect of size versus aggressive tendency on hierarchy maintenance (experiment 2).

(a) Observational experiment

   Experimental groups were video recorded in their respective home tanks immediately after amalgamation of same-sized individuals. Recordings occurred consistently on Monday, Wednesday, and Friday beginning at 1000h to avoid potential diurnal effects. Individual fish were identified by variations in banding and coloration. Each group was recorded for one hour on each recording day. Total length was recorded weekly on Mondays after video recordings using calipers. Each recorded video was scored for frequency of aggressive and submissive displays. Aggressive displays included biting, chasing, jolting, and lateral displays (Godwin 1994). Submissive displays included head shaking and retreating (Godwin 1994). Behavioral frequencies were used to construct rank-ordered hierarchies by means of David's Score dominance index (David 1987; Gammell *et al*. 2003), which was calculated for each individual with the formula:

   DS = % wins + % weighted wins – % losses – % weighted losses

Groups were recorded until hierarchies stabilized according to Spearman's correlation coefficient (r_s > 0.7) for at least three consecutive weeks. Fish that died during the observation period were not replaced. Observations began for cohort 1 in late July 2013 and for cohort 2 in early August 2014.

(b) Novel size-matched conspecific and novel heterospecific boldness assays

I measured individual responses towards a novel size-matched conspecific via simulation through the use of the mirror test (Budaev *et al*. 1999). This assay was performed only after at least 2 consecutive weeks of hierarchy stability. In this assay, focal fish were placed in a center compartment of a modified 38 liter aquarium (length x width x depth: 51 cm x 28 cm x 33 cm) that was divided into three equal-sized partitions separated by opaque black dividers, which included a central acclimation zone and two lateral stimulus presentation zones. The testing aquarium was also obscured from outside visual stimulation. A mirror was placed against the lateral wall of one of the stimulus zones for each trial. After 15-minute acclimation, both opaque dividers were removed and focal fish were video recorded for 15-minute sessions. The mirror was shifted to the opposite side for each consecutive trial to control for positional bias. Video recordings were analyzed for frequency of aggressive displays to the mirror stimulus and also for latency to enter and time spent in four zones in the testing arena (distal – on opposite side of stimulus, mid-tank, proximity – within two body lengths of stimulus, inspection – within 1.5 body lengths of stimulus).

I measured boldness by repeating the procedure used in the conspecific mirror assay with a live heterospecific stimulus in place of a mirror (Budaev *et al*. 1999). An adult male convict cichlid (*Amatitlania nigrofasciata*, TL = 61 mm) was used due to its complete novelty to a marine fish and also its size, which was not large enough to invoke a threatening predatory stimulation. The assay was

performed with the convict cichlid held in a glass jar (length x width x depth: 11 cm x 8 cm x 20 cm) filled with freshwater and placed in the stimulus zones as described earlier.

(c) Assessment of aggression via insertion of cohort 2 fish into cohort 1 groups

I performed the observational experiment (a) with a second cohort in July 2014 that yielded hierarchy stabilization within one week of group establishment due to higher aggressiveness   compared to the initial cohort. The more aggressive yet smaller fish provided an opportunity to test whether dominance would be relayed as an effect of asymmetry in body size or aggressiveness. After hierarchy stabilization was recorded for three consecutive weeks in the second cohort, I chose 4 top-ranked fish from cohort 2 (average TL = 35.5 +/- 0.5 mm) and introduced them individually to 4 groups from cohort 1 (average TL = 52 +/- 0.5 mm) that had already established stable hierarchies in their respective home tanks. These manipulated groups were video recorded on Monday, Wednesday, and Friday for 4 weeks. Cohort 2 fish were removed from cohort 1 home tanks following the recording period to avoid death/injury as a result of aggression.

Ethics Statement

All experimental protocols were executed in accordance with policies and guidelines for animal research instituted by the University of Windsor and the

Canadian Council on Animal Care under the Animal Utilization Project Proposal #11-17 and #14-12.

Data Analysis

I constructed weekly rank-order hierarchies using David's Score, which considers weighted wins and losses based off of dyadic encounters from the same week. I assessed hierarchy stability between weeks using the non-parametric Spearman's rank-order correlation coefficient. The use of Spearman's correlation coefficient as a metric of stability was adapted from Oliveira and Almada (1996), in which hierarchies were considered stable if the correlation coefficient (r_s) was equal to or greater than 0.7 as that would account for upwards of 50% of the total variance (r_s represents proportion of shared variance/correlation of determination). However, this analysis served strictly as a descriptive measure rather than an assessment of statistical probability. I attributed rank orders constructed from data collected during the week 26 to 28 observation period of the first cohort to each fish across the entirety of the observational experiment regardless of prior ranking. For each ranking across all four groups, I calculated the mean aggressive frequency per week and plotted these values across time. Spearman's rank-order correlation was also employed to assess the relationship between rank order and total length as well as the effect of rank on boldness, but was used conventionally to test probabilities. All statistical analyses were executed using SPSS Statistics 21 (IBM, New York, USA).

Results

Cohort 1

(a) Observational experiment

Experimental groups initiated and observed from July 2013 onward yielded stable dominance hierarchies within at least 26 weeks of group establishment (Figure 2.1). Group 1 (Figure 2.1a) experienced frequent occurrence of rank reordering, with some instances of between-week stability reaching $r_s = -1.000$. Group 2 (Figure 2.1b) had achieved social stasis ($r_s = 1.000$) between six consecutive weeks (weeks 6 to 12) due to zero occurrences of agonistic interactions that also prevented generation of rank orders. Three of the four groups (Figures 2.1a, b, c) experienced at least one large rank-shifting event with low-ranked fish displacing high-ranked fish. This social reversal is consistent in visualizations of rank and stability in Figures 2.1 and 2.2. Rank shifts occurred at week 15 for group 1 (Figure 2.1a and Figure 2.2a), week 14 for group 2 (Figure 2.1b and Figure 2.2b), and week 3 for group 3 (Figure 2.1c and Figure 2.2c). All four groups produced stable stratified rank orders by at least 26 weeks of group establishment (Figure 2.2), with rank changes occurring only in one group between two low-ranked fish in observations between weeks 26 to 28 (Figure 2.1c).

Visual comparison of rank and total length (Figure 2.3) depicts rank reversals between fish that concurrently retained high dominance rank and larger body size and fish that were smaller and previously subordinate. In two groups

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(group 2 – week 4: Figure 2.4b, group 4 – week 3: Figure 2.4d), there was rank

stabilization early but in both cases the dominant fish was not the largest

individual (Figure 2.3). In the other two groups, there was little difference in

rank or length throughout the first 17 weeks, although a dominance hierarchy did

stabilize by the end of observation (Figure 2.3). Throughout the 28 weeks of total

observation there was a large degree of variation in the correlation between rank

order and total length but by the final stabilization periods only group 4 showed a

marginally non-significant correlation between rank and total length, driven

predominantly by two dominant individuals (Figure 2.4d). The remaining groups

demonstrated stratification of a size hierarchy, albeit not reflecting the dominance

hierarchy nor a significant relationship between total length and rank.

While hierarchy stability and emergence of a prospective correlation

between total length and rank order did not occur until at least week 26, mean

frequency of aggressive behaviors combined from all four experimental groups

from approximately week 1 through 4 of observation appeared to provide an

accurate prediction of rank orders post-stabilization (Figure 2.5). Individuals that

were more aggressive from week 1 on yielded higher dominance rankings after

hierarchy stabilization, but this was driven largely by rank 1 and 2 fish.

(b) Novel size-matched conspecific and novel heterospecific boldness assays

No aggressive responses were recorded during the conspecific mirror test.

Hence, time spent in predefined preference zones was used as the metric of an

individual's propensity to investigate a novel conspecific carrying identical physical characteristics. Specifically in the inspection zone (within 1.5 body lengths of the stimulus), rank established from the observational experiment was not related to boldness in a conspecific stimulus context (r_s = -0.109, P = 0.677) (Figure 2.6a). Rank was also not related to boldness in a heterospecific stimulus context (r_s = 0.017, P = 0.950) (Figure 2.6b).

Cohort 2

(a) Observational experiment

Five experimental groups observed from August 2014 onward yielded stable dominance hierarchies immediately upon establishment at week 1 (r_s > 0.700) (Figure 2.7) and maintained stability for four consecutive weeks. Higher aggression between shoal mates resulted in great incidence of injury and death during the observation period. I halted the observation experiment after four weeks due to dwindling group sizes as a result of high intensity aggression causing death. A novel "hyper-aggressive" behavior not seen in cohort 1 observations was recorded during the four-week observation of cohort 2. This behavior, in which one fish bit the caudal fin of another and swam backwards, was performed expressly by highly aggressive dominant fish. Submissive displays were rare as aggressive dominants attacked regardless of reciprocated response.

(b) Assessment of aggression via insertion of cohort 2 fish into cohort 1 groups

In three of the four manipulated groups, the smaller fish from cohort 2 inserted into cohort 1 achieved rank 1 dominance status over larger fish from cohort 1 that were established in stable hierarchies approximately 9 months prior (Figure 2.8a, b, c) while in the fourth group (Figure 2.8d) the introduced fish achieved dominance rank 2. The contemporarily dominant and top ranked cohort 2 fish in the manipulated shoals deposed previously socially dominant and largest fish from cohort 1. These previously dominant fish were subordinated to rank 2 across the three groups. The newly recruited cohort 2 fish in cohort 1 group 1 attained rank 2 while still overcoming fish larger than itself during the observation period. Across all four manipulated groups, long-established rank orders from cohort 1 experienced consistent reordering until at least week 4 of the observation period.

Discussion

Previous studies exploring social hierarchies of cooperatively breeding hermaphroditic fishes have largely focused on pre-existing size hierarchies and corresponding dominance rank orders in wild populations (Forrester 1991; Buston 2003; Buston 2006; Ang and Manica 2010). Through the investigation of behavioral drivers underlying hierarchical stratification of rank and size in *A. ocellaris* by removing variation in body size at the initial point of group establishment, my study provides evidence that social dominance is driven predominantly by individual variation in aggressiveness rather than size.

In my study, neither experiment on hierarchy establishment over time (cohort 1) nor testing the role of aggression in stable hierarchies (cohort 2) provided evidence demonstrating that social dominance was based on size differences. Previous work on the proximate mechanisms associated with size hierarchies in cooperatively breeding hermaphroditic marine fishes has suggested that size hierarchies are emergent properties of aggression or resource interception (Fricke and Fricke 1977; Fricke 1979; Aldenhoven 1986; Munday 2002; Rubenstein and Kealey 2010) by dominants towards subordinates. However, these studies assessed regulation of size hierarchies in groups with established dominance hierarchies as opposed to regulation of social dominance by alternative proximate mechanisms. My study provides a novel approach in the investigation of size-matched groups in a closed system. The initial exclusion of variation in total length typically implicated in hierarchy organization presented experimental groups with the need to employ an alternative competitive asymmetry to facilitate dominance hierarchy formation and maintenance. Even with emergent size differences, size did not predict social dominance in either cohort. Hence, while size hierarchies may eventually be reflective of dominance hierarchies in established groups as documented in previous studies (Fricke and Fricke 1977; Forrester 1991; Buston 2003; Heg *et al.* 2004), my study provides clear evidence suggesting that *A. ocellaris* dominance hierarchies are not derived from size differences.

Results from both experimental cohorts in the current study were consistent with behavioural investigations of other gregarious reef fishes in which dominance in established groups was also associated with higher frequency of aggressive displays (*Amphiprion perideraion*: Allen 1972; *A. akallopisos*: Fricke 1979: *Dascyllys albisella*: Booth 1995). Additionally, my data substantiate effects described in previous studies that suggest that (1) high initial aggressiveness alongside (2) initial wins that reinforced aggression in dominants translating into further wins contributed strongly to the eventual rank orders of established hierarchies across taxa (Bonabeau *et al.* 1995; Bonabeau *et al.* 1997; Goessmann *et al*. 2000; Couzin and Krause 2003; Sumpter 2006). These relationships lend themselves to the self-structuring nature of dominance hierarchies in which positive feedback via aggression-driven dominance facilitates the formation and maintenance of stratified social structures.

The differential latency to hierarchy establishment between cohorts 1 and 2 may be attributed to aggression-driven mechanisms described in previous studies to be critical in the determination of stable rank orders (Chase 1982; Oliveira and Almada 1996; Chase *et al*. 2002). In the context of lower aggression in cohort 1, lengthy stabilization time may be reflective of the continuous assessment hypothesis (Enquist and Leimar 1983) in which rank orders are the consequent product of a protracted series of agonistic interactions that allow for improvement of individual assessment of relative competitive ability over time. Considering the numerous rank reversals that occurred throughout the

establishment period in cohort 1, the persistent rank reordering was likely a direct effect of repeated behavioral assessments over numerous agonistic interactions. By comparison, hasty stabilization with high aggression in cohort 2 may be a manifestation of the suppression hypothesis (Drummond and Osorno 1992; Dugatkin 1997; Rubenstein and Kealey 2010) in which rank orders in stable dominance hierarchies are based on continual reinforcement of the outcome of initial agonistic interactions at earlier time points. Given the complete absence of rank reordering in cohort 2, agonistic suppression by dominants appears to be a viable explanation for the immediacy of hierarchy stabilization.

In many fish species, behavioral boldness is typically correlated with dominance (Dingemanse and Goede 2004; Dahlbom *et al*. 2011; Colleter and Brown 2011). In the current study, I considered boldness as individual variation in propensity to engage in risk-taking behaviour. Interestingly, my assays showed no significant effect of rank on time spent near novel conspecific nor novel heterospecific stimuli. It may be possible that the test fish were not old enough to produce any variation in behavioral attributes beyond social aggressiveness. Juvenile and adult fish demonstrate variation in boldness in the context of inspection of novel stimuli and spaces (Sih *et al*. 2014b). Gregarious species may show less variation in the context of behavioral boldness because group living confers benefits that solitary animals would otherwise need to engage in bold behaviors to achieve (Sih *et al*. 2004ab, 2014). Conversely, boldness in a group context can confer dominance (Colleter and Brown 2011; Dahlbom *et al*. 2011).

Hence, behavioral types in *A. ocellaris* may require investigation using established and stabilized groups, as well as time-course analysis of emergence of variation in behavioral types.

The failure of the mirror test to elicit any aggressive response may be attributed to a fundamental flaw in the mirror test itself. Recent discourse on behavioral assays involving conspecific visual stimuli have suggested that mirrors do not provide appropriate responses to aggressive displays (Elwood and Arnott 2013; Balzarini *et al*. 2014). For example, the convict cichlid typically engages in intrasexual aggressive interactions that involve one fish facing the tail end of the other (Balzarini *et al*. 2014). Likewise, *A. ocellaris* agonistic interactions are unlikely to result in a mirror response from the subject of an aggressive display. Because the mirror image cannot respond in a manner typical of conspecific aggressive interactions, it begs the question as to whether the mirror test is a test of aggression at all. Certainly, the mirror test can function in the instance of organisms that display agonistic behaviors that involve head on or mirrored responses (Drozds *et al*. 2006; May and Mercier 2007; Balzarini *et al*. 2014) and also provide a physically identical stimulus. However, my experiment, among others (e.g. Brawn 1961; Jennings 2012; Elwood and Arnott 2013; Balzarini *et al*. 2014), provides evidence against the validity of using the mirror test in boldness and aggressiveness assays if studying fish that typically do not receive a mirrored response to aggressive behaviors.

Beyond direct interaction, multimodal transitive inference presents a viable

alternative explanation for dynamics underlying social organization (Nakamaru

and Sasaki 2003; Bond *et al*. 2004; Grosenick *et al*. 2007). Previous work has

provided evidence indicating that social fishes can assess the competitive capacity

of potential aggressors through observation of extraneous dyadic interactions

within their own social groups (Oliveira *et al*. 1998; Doutrelant *et al*. 2001; Earley

and Dugatkin 2002). While encounters and attempts to achieve dominance

through direct agonistic interactions is a common and viable route, eavesdropping

allows for the exclusion of energetic costs and the risk of injury and death

(Oliveira *et al*. 1998). In both cohorts, whether or not rank reordering occurs

may be attributed to evaluation of competitive capabilities via eavesdropping. In

the context of emergent size differences between fish in cohort 1 prior to

hierarchy stabilization, rank reordering and the rise in rank of smaller subordinate

fish may have been associated with risk determination using socially acquired

information collected during assessment of the competitive capabilities of other

fish during observed agonistic interactions. Likewise, the absence of rank shifts

between size-matched fish of cohort 2 may be attributed to the socially visible

aggression between higher ranked fish that may have discouraged direct

interactions from subordinate fish, hence resulting in quicker hierarchy

stabilization. The occurrence of rank reordering and eventual achievement of

social dominance in the absence of size asymmetry suggests the use of alternative

information, which may have been transitive evaluation of the aggressiveness of

other fish in the group. Further, the multimodal nature of transitive inference

suggests that the assessment of potential opponents includes more than socially

acquired information via eavesdropping. Alongside direct experience, previous

work has found that male fish can assess the potential quality and competitive

capability of other males via pheromonal communication, which would result in

modulation of androgen expression and eventually affect behavior (Oliveira *et al*.

2002). Given the multimodality of transitive inference and risk assessment in the

context of social dominance, it is unlikely that *A. ocellaris* dominance hierarchies

are strictly size-based. While my study provides evidence for aggression-driven

dominance, additional avenues of social modulation including hormone control

should be studied.

In summary, my study found that social dominance is rewarded to more

aggressive fish in size-matched groups of *A. ocellaris*. These findings have

potentially wider implications on the notion of social hierarchies being regulated

predominantly by size differences between dominants and subordinates. Albeit

that size hierarchies coexist and typically correlate with social hierarchies (Buston

and Cant 2006), further consideration should be allocated to the competitive

drivers underpinning size variation as opposed to suggesting that size variation

drives and mediates social dominance.

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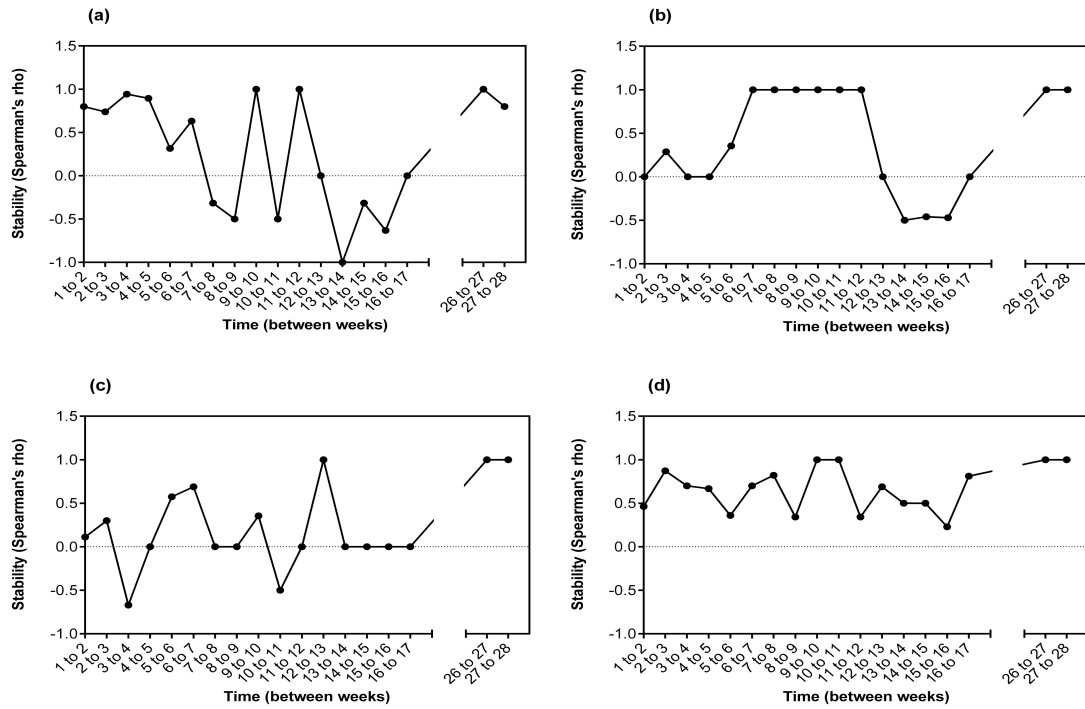
Figure 2.1: Dominance hierarchy stability between successive weeks of observation measured as Spearman's rank-order correlation per group (cohort 1). A hierarchy was considered stable between weeks if Spearman's rho $\geq$ 0.7.

Figure 2.2: Individual ordinal rankings in hierarchies over 28 weeks for four groups (cohort 1) determined via David's Score dominance index. Nominal designations (A-E) denote fish identity.

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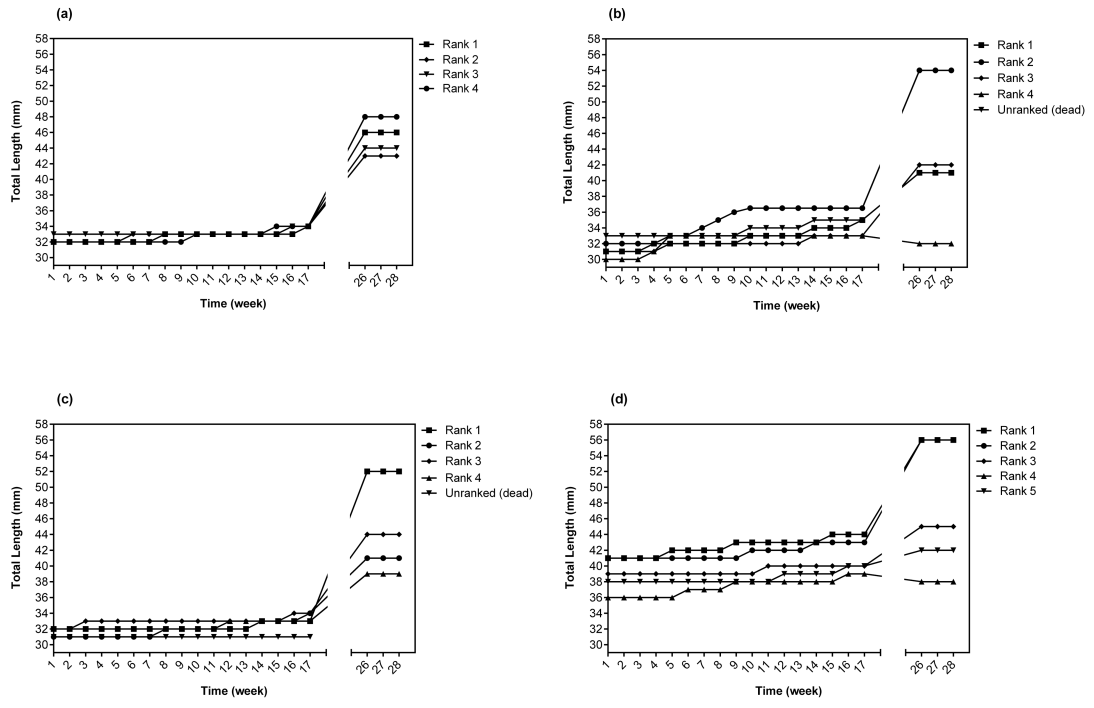
Figure 2.3: Total length of individual fish recorded per group (cohort 1) over the observation period.
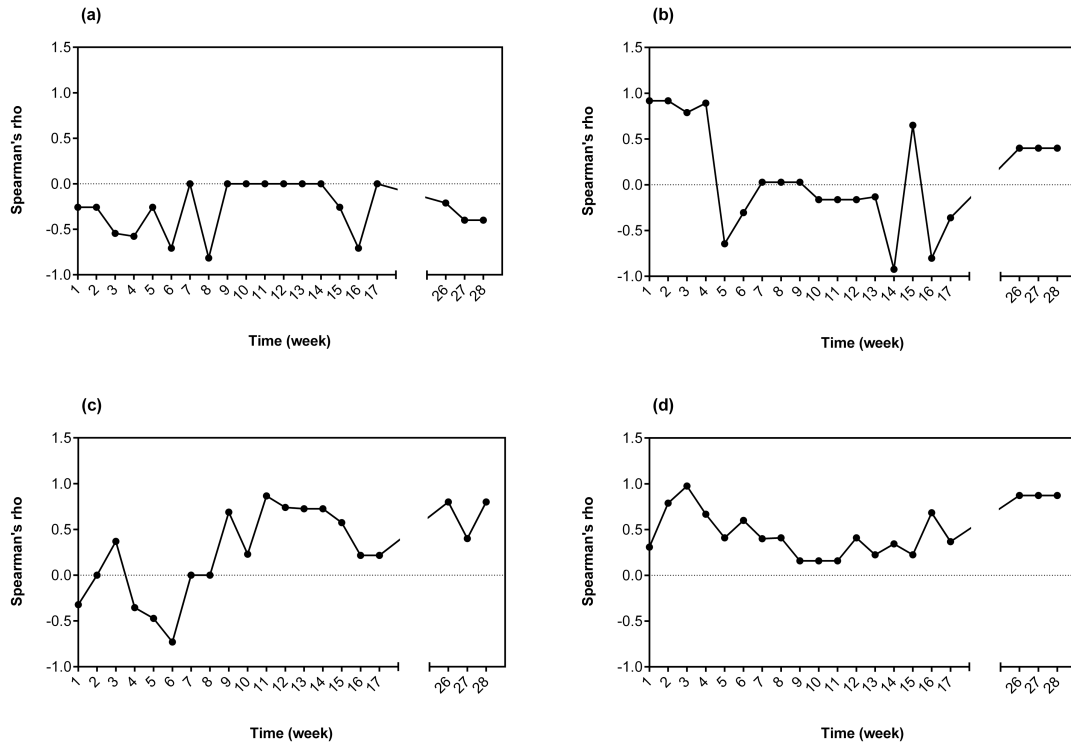
Figure 2.4: Correlation between dominance ranks to total length measured as Spearman's rank-order correlation per group (cohort 1) across the observation period.
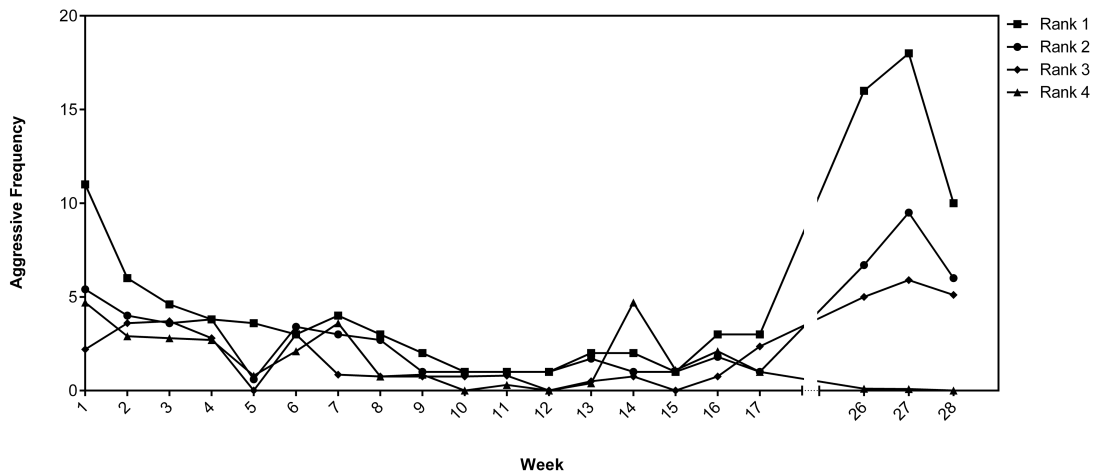
Figure 2.5: Mean aggression across observation period by ordinal ranks determined by follow-up observations (week 26-28). Highly variable across time, highly aggressive fish at earlier time-points appear to retain higher ranks after hierarchy establishment.
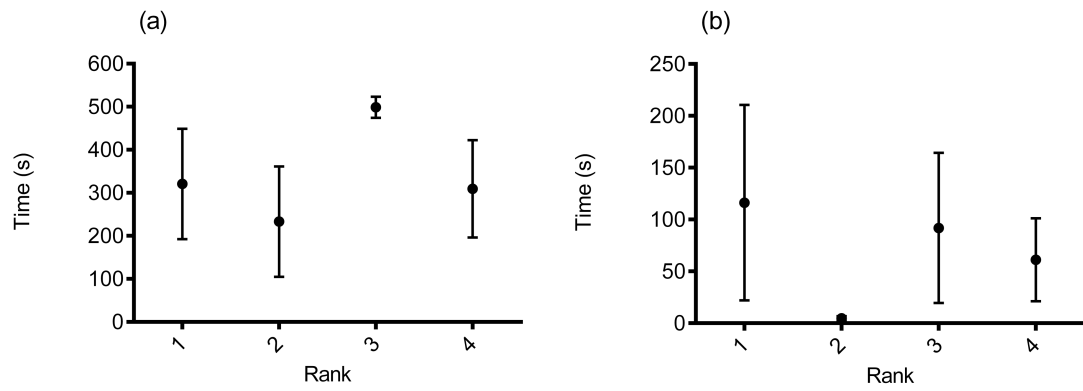
Figure 2.6: Mean ± SE time spent within 1.5 body lengths of a stimulus in (a) conspecific and (b) heterospecific contexts for individually ranked fish of four groups (cohort 1). Dominance ranks were determined via follow-up (week 26-28) observations.
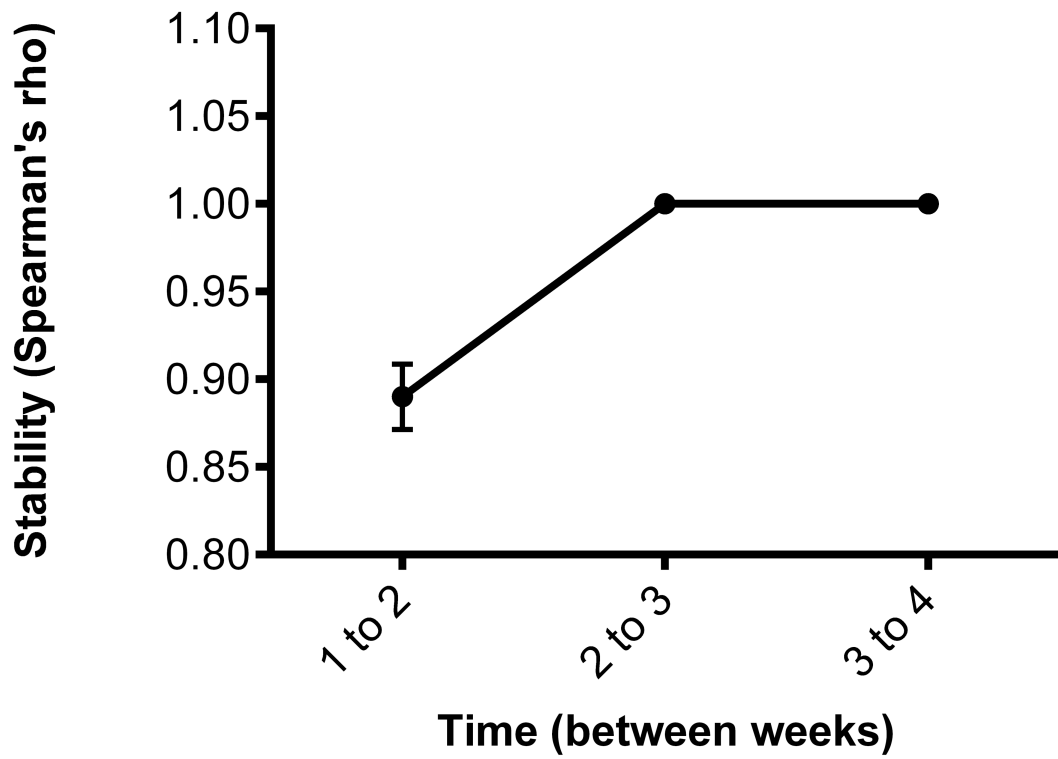
Figure 2.7: Dominance hierarchy stability measured as mean ± SE Spearman's rank-order correlation for five groups (cohort 2). A hierarchy was considered stable between weeks if Spearman's rho ≥ 0.7.
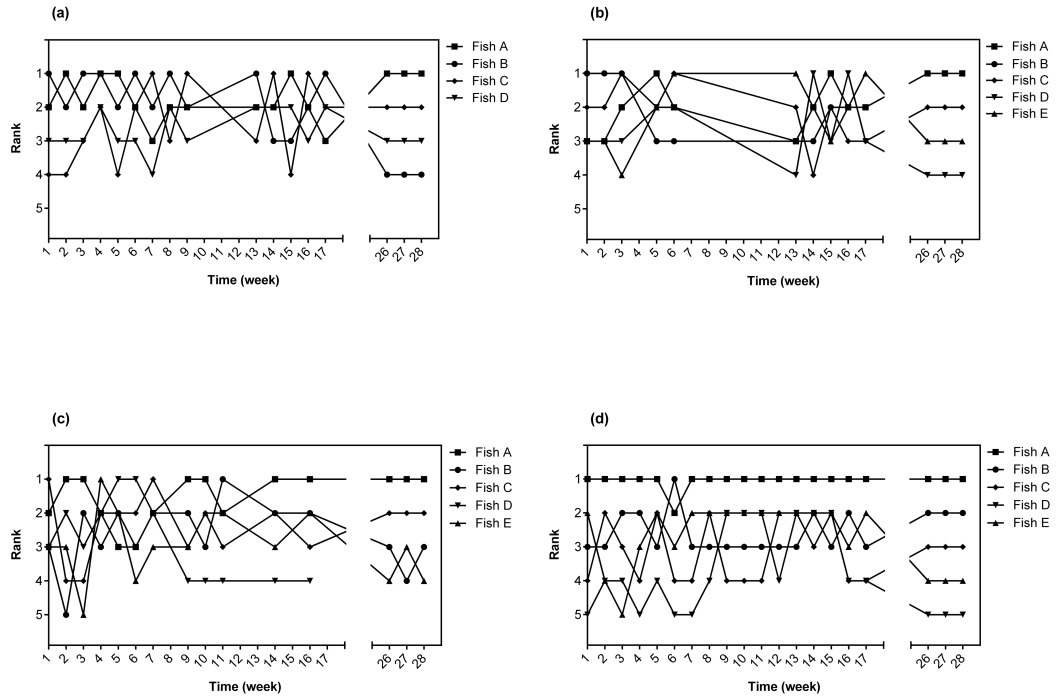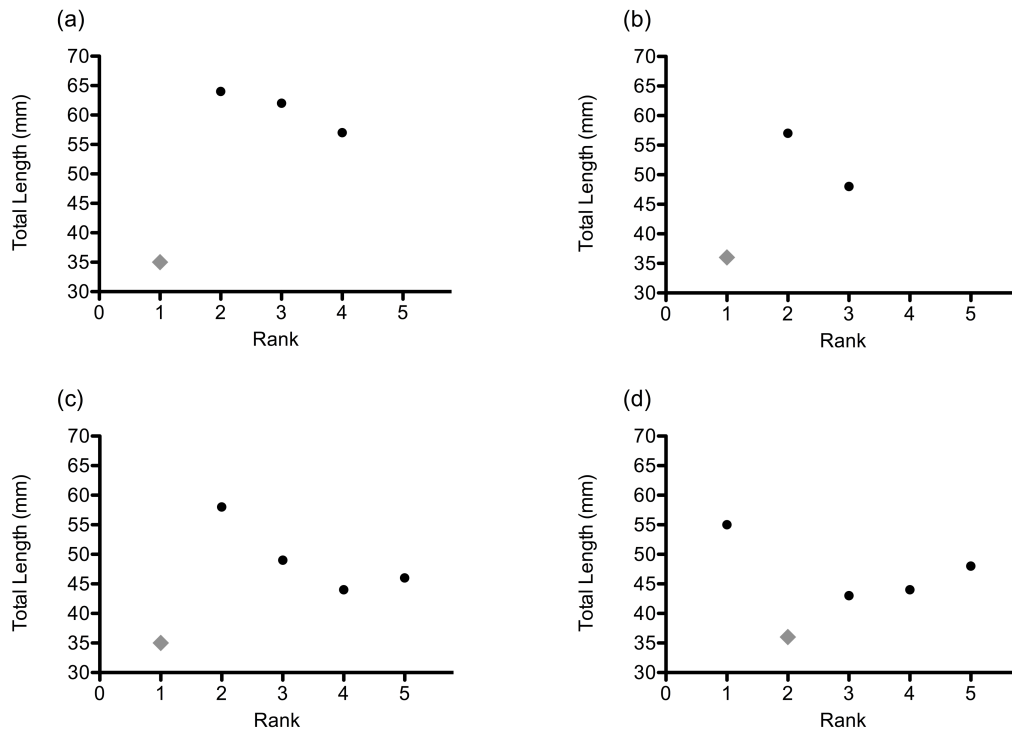
Figure 2.8: Individual total length recorded post-stabilization of hierarchies after recruitment of aggressive smaller fish (cohort 2, ◆) into previously-established hierarchies (cohort 1, ●).

CHAPTER III: DOMINANCE AND EXPRESSION PROFILES OF CORTISOL, 11-KETOTESTOSTERONE, AND TESTOSTERONE IN THE FALSE PERCULA ANEMONEFISH *AMPHIPRION OCELLARIS*

Introduction

Social animals form dominance hierarchies that facilitate the allocation of limited resources. The social ranks that denote dominance within these hierarchies are founded on a variety of competitive and noncompetitive asymmetries including aggressiveness (Alexander 1974; Alexander and Borgia 1978; Ellis 1995; Grosenick *et al*. 2007; Rubenstein and Kealey 2010), age (Drickamer and Vessey 1973; Saitou 1979; Hogstad 1989), recruitment seniority (Levin *et al*. 2000; Bond *et al.* 2004; Grosenick *et al*. 2007), and physical size (Buston 2003; Buston 2006; Bond *et al.* 2004; Grosenick *et al*. 2007). Previous work has shown a strong association between asymmetries that incur social dominance and androgen and glucocorticoid expression. Basal androgen expression is positively correlated with social dominance across taxa, particularly in the motivation of agonistic and reproductive behaviours (e.g., Hart 1974; Pall *et al*. 2002; Rhen and Crews 2002), and basal glucocorticoid expression varies across species, with some showing increasing expression in subordinates (e.g., Ejike and Schreck 1980; Abbott *et al.* 2003; Muller and Wrangham 2004), while others show heightened expression in dominants (e.g., Creel *et al.* 1996; Fox *et al*. 1997; Creel 2001). Chronic agonistic incursions from high-ranking dominants typically produce the stress of subordination (Blanchard *et al*. 1993; Devries *et al*. 2003). Conversely, persistent display of aggressive, territorial, and courtship

behaviours in high-ranking individuals results in the stress of dominance (e.g., Creel *et al.* 1996; Fox *et al*. 1997; Creel 2001). While glucocorticoid response to acute stressors is beneficial (Creel 2001; Devries *et al*. 2003), the persistence of elevated expression may also be advantageous in the context of social interactions that coincide with heightened androgen levels.

Anemonefishes are protandrous coral reef fishes that live in haremic aggregations in obligate symbiosis with sea anemones (Moyer and Nakazono 1978; Fautin and Allen 1992; Iwata *et al*. 2008). Groups are composed of a large dominant female, breeding dominant male, and subordinate non-breeding males (Fricke 1979; Buston 2003). Upon removal of the dominant female, the breeding male undergoes protandrous sex change while the highest-ranked subordinate non-breeder assumes the role of the breeding dominant male (Fricke and Fricke 1977; Buston 2003). In contrast to gonochoristic fishes, gonadal changes in the protandrous anemonefishes provide an advantage for studying gonadal steroid correlates of social dominance via reversal of typical sex roles within dominant fish, while rigid social communities allow for further exploration of the role of glucocorticoid expression on behavioural modulation in social contexts. The false percula anemonefish, *Amphiprion ocellaris,* is ideal for the laboratory study of behavioural endocrine relationships due to its small size, hardiness, and well-documented behavioural repertoire (Godwin 1994; Buston 2003).

The current study examines differences in cortisol, 11-ketotestosterone (11-KT), and testosterone (T) expression between dominant and subordinate

juvenile male *Amphiprion ocellaris* and the time course analysis of hormone

expression profiles resulting from eventual stratification of clear rank orders and

stabilization of dominance hierarchies. I examine the following predictions: (1)

basal 11-KT and T expression increases with social rank resulting in increased

aggressiveness and (2) basal cortisol expression is elevated in top-ranked

dominant and lowest-ranked subordinate fish as a consequence of socially-

modulated stress response.

Materials and Methods

Animals and housing

As described in Cheung & Higgs (2015, submitted; Chapter 2), captive-

bred 5-month-old juvenile male false percula anemonefish (*Amphiprion ocellaris*)

bred from an Indonesian wildstock were obtained from a regional supplier

(SeaUMarine, Ontario, Canada). Fish were housed in size-matched groups of 5 in

76 litre glass aquaria (length x width x depth: 77 cm x 33 cm x 33 cm) in the

animal housing facilities at the University of Windsor (Windsor, Ontario, Canada).

Dechlorinated tap water was supplemented with 30 g/L aquarium salt (Instant

Ocean – United Pet Group, Virginia, USA). Water was filtered using three-stage

filtration external power filters (Marineland – United Pet Group, Virginia, USA).

Water temperature was maintained at 26°C using thermostat-controlled aquarium

heaters (EHEIM GmbH & Co KG, Deizisau, Germany). A 12L:12D light cycle was

maintained with lights on at 0630h. Environmental enrichment was provided

through hiding spaces via a large rock, replica anemone, and 3 cm deep aragonite sand substrate. Fish were fed twice daily with a pulverized mixture of dry food composed of Tetramin flake (Tetra Corp., Melle, Germany) and dried brine shrimp (Hikari Fish Industry Group, Yamasaki, Japan). This diet was supplemented with weekly feedings of garlic-soaked defrosted brine shrimp cubes (*Artemia salina*, Hikari Fish Industry Group, Yamasaki, Japan). A total of 34 fish (n=34) were tested and included in analysis. These fish were divided into two experimental cohorts to evaluate behavioural properties driving dominance hierarchy establishment (chapter 2): cohort 1 – complaisant groups that achieved hierarchy stability over 28 weeks, cohort 2 – highly aggressive groups that achieved hierarchy stability within 1 day.

Shoal observation

Experimental groups were video recorded on Monday, Wednesday, and Friday at 1000h of each week. Each recording was one hour long. Recorded videos were scored for frequency of aggressive and submissive displays. Aggressive displays included biting, chasing, jolting, and lateral displays (Godwin 1994). Submissive displays included head shaking and retreating (Godwin 1994). Behavioural frequencies were used to construct rank-order dominance hierarchies using David's Score dominance index (David 1987; Gammell *et al*. 2003). This index was calculated for each individual using the following formula:

DS = % wins + % weighted wins – % losses – % weighted losses

Observations continued until hierarchies stabilized between weeks according to Spearman's correlation coefficient ($r_s > 0.7$: Oliveira and Almada 1996) for at least three consecutive weeks.

Sample collection

Water samples were collected on (cohort 1) January 13 (pre-hierarchy stabilization – pre-HS) and May 9, 2014 (post-hierarchy stabilization – post-HS) and (cohort 2) September 15 (pre-HS) and October 7, 2014 (post-HS). All samples were collected between 1100h and 1200h. Prior to use, 600 mL glass beakers were rinsed with anhydrous ethanol and reverse-osmosis filtered water. After rinsing, beakers were filled with 250mL of clean unused saltwater (reverse osmosis water supplemented with 30 g/L aquarium salt). Fish were removed from their home tanks and placed in individual beakers for 30 minutes. The beakers were obscured by a cardboard matrix to eliminate any external visual stimulation. After 30 minutes, fish were returned to their respective home tanks. Water samples were filtered to remove particulate matter and stored at -20°C until hormone extraction.

I employed the extraction, elution, and assay procedures described in Kidd *et al*. (2010) and manufacturer instructions provided by Cayman Chemical unless otherwise stated.

Extraction

Water samples were defrosted at 4°C overnight prior to extraction. For each extraction, the lower end of a Sep-Pack Plus C18 cartridge (Waters Limited – WAT023501, Ontario, Canada) was connected to a variable-flow chemical pump (Cole-Parmer, Illinois, USA) using chemical-resistant tubing. The upper end of the C18 cartridge was connected to a glass funnel, also using chemical-resistant tubing. A flow rate of 10 mL/min was maintained for all liquids processed through each C18 cartridge. Each cartridge was primed using 6 mL of EtOH, followed by 6 mL of reverse-osmosis filtered water. Each water sample was processed through a separate cartridge. After a water sample was completely processed, the cartridge was flushed with 6 mL of reverse-osmosis filtered water. Following extraction, cartridges were removed from the pump and funnel, covered with Parafilm (Bemis NA, Wisconsin, USA), and stored at -20°C until elution.

Elution

C18 cartridges were removed from the freezer and defrosted at room temperature for 30 minutes. Cartridges were then attached to an 18-port vacuum manifold and eluted with 4 mL of EtOAc into 7 mL glass scintillation vials. Vials containing eluted samples were placed under a fumehood to allow for evaporation of EtOAc solvent over three days. The remaining dried pellets were stored at -20°C until enzyme immunoassays were performed.

Enzyme immunoassay

Immediately prior to performing the enzyme immunoassay, samples were removed from the freezer and reconstituted with 1 mL of assay buffer. Reconstituted samples were divided into three 0.3 mL aliquots for the separate 11-KT, T, and cortisol assays. Commercial EIA kits were obtained from Cayman Chemical (cortisol #500360, 11-KT #582751, T #582701; Michigan, USA). For each kit, manufacturer instructions were followed as written in the provided long form kit booklets. Plates were read using a BioTek Multi-Mode Microplate reader (BioTek Instruments, Vermont, USA) at 412 nm.

Data analysis

Rank-order dominance hierarchies were constructed using the David's Score dominance index, which considers weighted wins and losses based off of dyadic encounters between individuals of the same group within the same week (David 1987; Gammell *et al*. 2003). Differences in log-transformed mean expression of cortisol, 11-KT, and T between dominance ranks were analysed separately for the two experimental cohorts by one-way ANOVA, followed by Tukey's HSD multiple comparison post-hoc analysis. Correlations between expression of cortisol, 11-KT, and T were analysed separately for the two experimental cohorts within ranks at each time-point using Spearman's rank-order correlation. Analyses for the two experimental cohorts were executed separately due to difference in time to hierarchy stabilization and the largely divergent

behavioural properties of each cohort. All statistical analyses were executed using SPSS Statistics 21 (IBM, New York, USA). Data visualizations were produced using GraphPad Prism 6.01 (GraphPad Software, Inc., California, USA).

Results

Dominance Rank and Cortisol Expression

In cohort 1, water cortisol concentration differed significantly between dominance ranks during the pre-HS stage (Figure 3.1a, one-way ANOVA, $F_{(3,13)}$ = 5.413, $p < 0.05$). Tukey's post-hoc analysis revealed a significant difference between rank 1 and rank 2 fish ($p < 0.01$), and marginally nonsignificant differences when comparing rank 1 to ranks 3 ($p = 0.068$) and 4 ($p = 0.062$). Cortisol concentration did not differ significantly between dominance ranks post-HS (Figure 3.1b, one-way ANOVA, $F_{(3,13)}$ = 1.740, $p > 0.05$). However, rank appeared to suggest a hormetic effect on cortisol expression, with heightened titres occurring in dominant rank 1 fish and subordinate rank 4 fish and lower titres occurring in mid-ranks 2 and 3 (Figure 3.1b).

In cohort 2, water cortisol concentration differed significantly between dominance ranks during the pre-HS stage (Figure 3.1c, one-way ANOVA, $F_{(3,13)}$ = 6.834, $p < 0.01$). Post-hoc analysis revealed a significant difference when comparing subordinate rank 4 fish to rank 1, rank 2, and rank 3 ($p < 0.05$). Cortisol concentration did not differ significantly between dominance ranks after hierarchy stabilization (Figure 3.1d, one-way ANOVA, $F_{(2,8)}$ = 1.742, $p > 0.05$).

Dominance Rank and 11-KT Expression

Water 11-KT concentration did not differ significantly between dominance ranks at either time point for each cohort (Figure 3.2). In cohort 1, 11-KT levels demonstrated a stepwise pattern of decline from rank 1 onward, except when exceeded by rank 2 fish at post-HS (Figure 3.2b). This pattern was not reflected in cohort 2, in which rank 1 fish retained larger 11-KT levels across both time points, albeit with negligible differences between ranks 1 and 2 at post-HS.

Dominance Rank and T Expression

Water T concentration did not differ significantly between dominance ranks at either time point in cohort 1 (Figure 3.3a, 3.3b). At pre-HS, T concentration appeared heightened in rank 1 fish however, hierarchy stabilization at post-HS resulted in subordinate fish demonstrating a pattern of increased T levels relative to rank 1 dominants in the post-HS stage, although neither pattern reached the level of statistical significance.

In cohort 2, water T concentration differed significantly between dominance ranks during the pre-HS stage (Figure 3.3c, one-way ANOVA, $F_{(3,13)}$ = 4.502, $p < 0.05$). Post-hoc analysis revealed a significant difference between fish of ranks 1 and 4 ($p < 0.05$). At post-HS, dominance rank demonstrated a suggestive effect on water T concentration (Figure 3.3d, one-way ANOVA, $F_{(2,8)}$ = 3.908, $p = 0.065$). Across both time points, T concentrations declined in a stepwise pattern with highest mean concentrations recorded for rank 1.

Correlation between Expression of Cortisol, 11-KT, and T

In cohort 1 at the pre-HS stage, cortisol levels were correlated within dominance ranks with 11-KT (cortisol and 11-KT, $r_s = 0.517$, $p < 0.05$) and T (cortisol and T, $r_s = 0.679$, $p < 0.01$), while 11-KT was correlated with T levels (11-KT and T, $r_s = 0.784$, $p < 0.05$). At the post-HS stage, 11-KT was again correlated with T (11-KT and T, $r_s = 0.865$, $p < 0.01$). Cortisol yielded a marginally non-significant correlation with 11-KT post-HS (cortisol and 11-KT, $r_s = 0.466$, $p = 0.06$). Significant correlations between cortisol, 11-KT, and T were not observed for cohort 2 at either time-point.

Discussion

My data show that waterborne expression of cortisol in *A. ocellaris* is largely dependent on individual social status and stability of the social hierarchy within which an individual resides. My results from the pre-hierarchy stabilization social stage also corroborate previous studies of androgen profiles in gregarious fishes in which top-ranked dominants express higher androgen levels than their submissive counterparts (Maruska and Fernald 2006; Parikh *et al*. 2006; Burmeister *et al*. 2007; Maruska *et al*. 2011), although in the current study the trends were not statistically significant. Further, my results support previous reports (Fox *et al.* 1997; Creel 2001; Hofmann and Fernald 2001) on the relationship between heightened cortisol expression in top-ranked dominants and lowest-ranked subordinates. Collectively, my study suggests that the expression

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profiles of cortisol may serve as endogenous representations of individual social status within both unestablished and stable dominance hierarchies, as well as in variable contexts per latency to hierarchy stabilization and overall aggressiveness of individual communities.

My results show that androgen levels were typically highest in top-ranked dominants except in cohort 1 at the post-HS stage. Generally, this is consistent with previous studies of gregarious fishes that have documented apical expression of both circulating and waterborne androgens in socially dominant individuals (e.g., *Astatotilapia burtoni*: Parikh *et al.* 2006, Marusksa and Fernald 2010; *Oreochromis mossambicus*: Oliveira *et al*. 1996, Barata *et al*. 2007; *Neolamprologus pulcher*: Desjardins *et al.* 2008, Taves *et al*. 2009; *Danio rerio*: Filby *et al*. 2010). The relationship between heightened androgen expression and dominance rank is also consistent with previously reported relationships between aggression, androgen expression profiles, and social status (Oliveira *et al*. 2001; Oliveira *et al*. 2002). In novel unstable hierarchies, contention for dominance status is inevitably coupled with heightened aggression and correspondingly high androgen levels (Oliveira and Almada 1998; Oliveira *et al*. 2001). While aggression in stable hierarchies may be lower, dominants have been recorded to retain elevated aggressiveness and androgen levels in order to maintain dominance status obtained during times of social instability (Oliveira *et al*. 1996; Oliveira and Almada 1998; Parikh *et al*. 2006). Although heightened androgen expression in variable contexts is expected in socially dominant males of gregarious

gonochoristic fishes, the same cannot be expected for a protandrous hermaphrodite without validation of female, male, and transitional hormone expression profiles. In cohort 1 at the post-HS stage, 11-KT and T levels were notably higher for rank 2 individuals than rank 1. The structure and function of anemonefish communities provides potential explanation via the occurrence of protandrous sex change. In several protandrous reef fishes (e.g., Warner and Swearer 1991; Godwin and Thomas 1993; Godwin 1994), the onset of sex change typically coincides with declining androgen levels. Beyond behavioural and hormonal differences, top-ranked females can then retain dominance via alternative means including physical asymmetries (Alexander 1974; Alexander and Borgia 1978; Ellis 1995; Buston 2003; Grosenick *et al*. 2007; Rubenstein and Kealey 2010) and the threat of expulsion (Wong *et al*. 2007; Raihani *et al*. 2012). However, in the absence of sex validation of rank 1 fish studied in the current investigation, further work will be required on excision and identification of testicular and ovarian tissue to verify individual fish sex.

Elevated androgen expression in rank 2 individuals may be associated with the need to retain social status as dominant breeding male over subordinate non-breeders via aggressive behaviours, as documented in cooperatively-breeding species (e.g., *N. pulcher*, Taves *et al*. 2009). However, latency to stabilization may allowed for the potential occurrence of protandrous sex change in cohort 1 (albeit this requires validation via dissection). It is important to note that cohort 1 stabilized over 28 weeks, while cohort 2 stabilized with near-immediacy. The

longer time to stabilization may have coincided with the potential definition of eventual reproductive roles, while immediate stabilization would not have done so.

In both cohorts, androgen expression in subordinates of ranks 2 to 4 was not substantially variable with the primary exception of cohort 1 post-HS, perhaps due to the potential occurrence of protandrous sex change during hierarchy stabilization. While top-ranked dominants are expected to express higher androgen levels in order to retain social status via aggressive behaviours, the elevation of rank for non-breeding males in a queued breeding system such as that of *A. ocellaris* may not be heavily reliant on behavioural modulation via androgen expression, instead occurring via removal of higher-ranked individuals. Previous work on gregarious cichlids (e.g., *A. burtoni*, Parikh *et al*. 2006; *A. nigrofasciata*, Sessa *et al*. 2013; *N. pulcher*, Taves *et al*. 2009) has provided evidence for a similar phenomenon in which reproductive males expressed higher androgen levels while subordinate males did not demonstrate considerable variation in androgen expression between each other. Hence, variation in androgen expression in subordinates is not likely to be expected due to the mechanism through which social status changes within established breeding queues.

Distinctly variable stress profiles of individual ranks across both cohorts and time points suggest that cortisol expression is likely representative of ongoing social dynamics underlying dominance hierarchy function, as well as individual

social status. In both stable and unstable hierarchies, top-ranked dominants and lowest-ranked subordinates persistently expressed high cortisol levels with the exception of rank 4 in cohort 1 at pre-HS. Heightened cortisol expression in dominants is not intuitively explicable because of the implicit assumption that social subordination is correlated with a potent stress response. Previous work has documented increased glucocorticoid expression in social dominants across a variety of gregarious species (e.g., Creel *et al.* 1996; Fox *et al*. 1997; Creel 2001). While stress of subordination is expected to be associated with agonistic victimization and losses in dyadic encounters, the stress of dominance is likely to be identified with social status acquisition and retention via aggressive behaviours (Creel 2001). Hence, the occurrence of heightened cortisol levels in rank 1 dominants and rank 4 subordinates may be consistent with previous reports of dominance and subordination-induced stress (Ejike and Schreck 1980; Creel *et al.* 1996; Fox *et al*. 1997; Creel 2001 Abbott *et al*. 2003; Muller and Wrangham 2004).

Mean cortisol levels for rank 2 fish were persistently lower in both stable and unstable contexts for both cohorts. While lower cortisol expression in a stable social setting is expected due to overall reduction of within-group aggression for status acquisition, low rank 2 cortisol expression during hierarchy instability is not entirely comprehensible when considered in the social context and androgen expression profile. There are a multitude of examples reporting elevated investment into status acquisition efforts during times of social instability in

gregarious fishes predominantly due to fitness benefits incurred by achieving social dominance (e.g., Robinson 1986; Majolo *et al*. 2012). Lek-breeding cichlids, such as *A. burtoni* and *O. mossambicus*, convey two notable behavioural phenotypes: territorial and non-territorial (Oliveira *et al*. 2002; Parikh *et al*. 2006). Energetic investment into achieving territorial status can translate into more breeding opportunities, as well as overall preferential access to food and living space, while non-territorials are subsequently subordinated in their access to mates and limited resources (White *et al*. 2002). In the current study, the combination of size-matched *A. ocellaris* into novel shoals should harbor the expectation of increased cortisol expression particularly from rank 2 individuals that may be competing for both top-ranked dominance status, which can translate into protandrous sex change, as well as suppressing behaviourally submissive individuals into lower ranks so that position as top-ranked breeding male can be retained. A potential interpretation for these data is that rank 2 individuals are inferring the expected ranks of other individuals within the same group by observation of agonistic interactions occurring during periods of social instability. Via transitive inference, a reduction in direct behaviourally aggressive investment into status acquisition can allow for energetic investment into reproductive efforts instead (Bond *et al*. 2004; Grosenick *et al*. 2007). This explanation is compatible with the androgen expression profiles for both cohorts in which rank 2 11-KT and T expression was similar to that of rank 3 and 4 subordinates with the exception of the potentially female-dominated cohort 1 at post-HS.

Correlations between 11-KT and T recorded in both the pre-HS and post-HS stages are consistent with reports from previous investigations of hormonal correlates of social behaviour in teleosts (e.g., Oliveira *et al.* 1996; Carlson *et al.* 2000; Parikh *et al*. 2006). Additionally, this relationship is compatible with previous studies that provide evidence suggesting that both androgens, 11-KT and T, are related to elevated aggressiveness within individuals (e.g., Oliveira *et al*. 2002; Oliveira *et al*. 2009; Taves *et al*. 2009). The correlations observed between cortisol and 11-KT, as well as cortisol and T, have been reported in other teleosts (e.g., *Xiphophorus helleri*, Oliveira *et al.* 2002). It has been suggested that this relationship may be affected by social modulation of androgen levels and consequently, reproductive capacity (Creel 2001; Oliveira *et al*. 2009). However, the absence of this relationship at the post-HS stage may require further study to elucidate conjunctive effects of cortisol and androgen expression on social status.

The expectation of stress profiles responding to overall aggressiveness within groups can be substantiated by elevated cortisol expression by rank 4 individuals in cohort 2 at pre-HS. The data was divided into two separate cohorts due to the distinct behavioural properties of each cohort. Shoals in cohort 2 were substantially more aggressive than those of cohort 1 resulting in a near immediacy of hierarchy stabilization and numerous deaths due to aggressive behaviours directed to submissive individuals by highly aggressive dominants. Hence, the elevated cortisol expression levels of rank 4 individuals in cohort 2 at pre-HS can be directly attributed to characteristic stress of subordination (Creel *et al*. 1996;

Creel 2001). Additionally, cortisol expression of rank 4 subordinates may have also been exacerbated by exclusion from resource access (Wong *et al.*, 2008) and the threat of punishment (Cant and Johnstone 2006; Wong *et al.* 2007) as a direct result of agonistic suppression by social dominants. Overall, stress profiles may not be useful in indicating an individual's social nor reproductive state considering the potential overlap and similarity of cortisol levels between highest-ranked dominants and lowest-ranked subordinates.

In his review of social dominance and variation in stress profiles, Creel (2001) describes the typically dichotomous relationship between stress expression profiles in cooperative and non-cooperative breeders. In cooperative breeding arrangements, dominants typically have highest glucocorticoid expression. By comparison, subordinates in non-cooperatively breeding arrangements retain the highest glucocorticoid expression (Creel 2001). Interestingly, there is mounting evidence suggesting that there can be within-species variation in stress profiles modulated by individual dominance or subordination status, in which sex and variable social and breeding contexts can affect whether a conspecific aggregation reflects the stress of dominance, stress of subordination, or both (Creel 2001). In the current study, the data reflect within-species variation in cortisol expression between the two contexts of social stability. Other species, such as the Florida scrub jay *Aphelocma coerulescens*, demonstrate mating stage-dependent variation in dominant versus subordinate levels of glucocorticoid expression (Mays *et al.* 1991). These studies incorporate long-term measurement of hormone expression

profiles, allowing for elucidation of stage-dependent stress profiles. Hence, it may be necessary to consider the temporal scale at which studies assessing individual variation in hormone expression profiles are performed, as extended studies may provide further evidence standing divergent with the conventionally dichotomous view of stress profile variation both between and within species.

The challenge hypothesis is an avian paradigm that has been suggested for application to teleost systems when considering variation in hormone expression profiles between variable contexts of social stability (Wingfield *et al*. 1990). During social instability and heightened aggression, the challenge hypothesis indicates that androgen expression profiles are reflective of an individual's eventual social status in a stable social setting (Wingfield *et al*. 1990). Consequently, higher androgen levels would be expected in dominants in socially stable settings when compared to their subordinate counterparts (Wingfield *et al*. 1990). In the current study, occurrence of high androgen levels in rank 1 dominants in unstable hierarchies provides some substantiation for the applicability of the challenge hypothesis in teleost models due to retention of social status by the same dominants in periods of both social instability and stability. Hence, initial elevated androgen expression can be predictive of an individual's eventual dominance rank. However, the validity of applying the challenge hypothesis to non-avian models has been challenged due to its dependency on seasonal mating cycles (Desjardins *et al*. 2006; Parikh *et al*. 2006). Nonetheless, the framework detailing androgen profiles as an accurate

representation of aggression and dominance in gregarious animals may provide universal applicability of the challenge hypothesis beyond the constraints initially detailed for seasonal breeding patterns of avian models.

In summary, my study found that cortisol expression profiles are reflective of individual social status and within-group stability of dominance hierarchies. These findings substantiate previous reports that variable hormone expression can contribute to endogenous control of individual social status (Oliveira *et* al. 1996; Hirschenhauser and Oliveira 2006; Parikh *et al*. 2006), as well as the status of others within the same group via behavioural modulation (Oliveira *et al*. 1996; Pall *et al*. 2002; Rhen and Crews 2002). Further consideration should be given to experimental manipulation of social contexts once individual basal expression profiles are recorded, which may allow for the elucidation of fundamental relationships between basal hormone expression, aggression, and social status.

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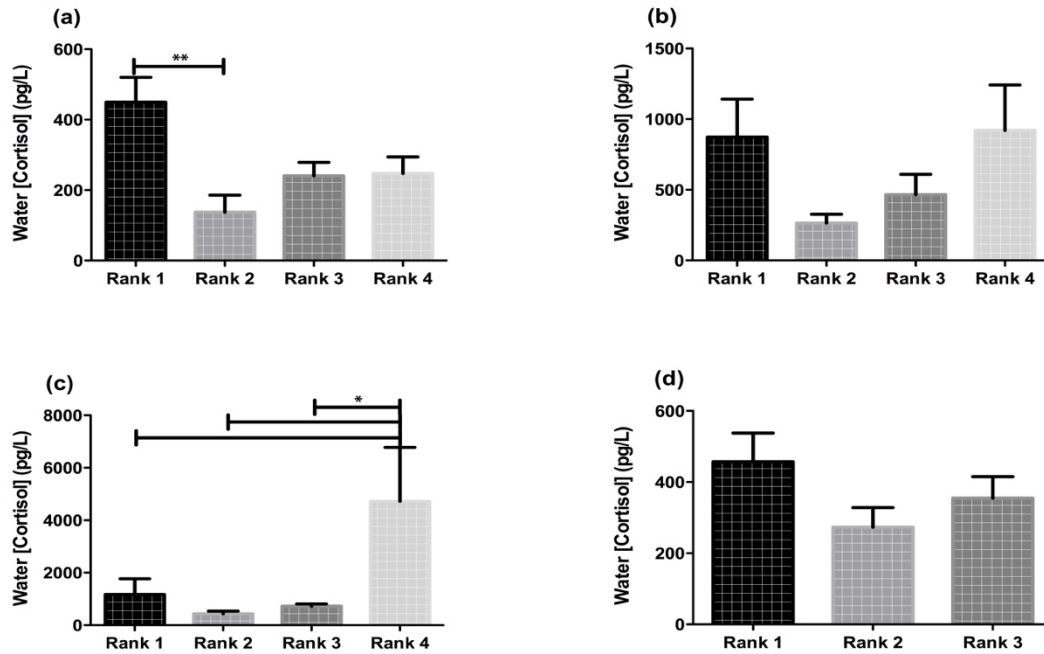# Figures and Figure Legends

Figure 3.1: Mean $\pm$ SE concentration of waterborne cortisol within dominance ranks determined via David's Score dominance index. (a) Cohort 1 pre-hierarchy stabilization, (b) Cohort 1 post-hierarchy stabilization, (c) Cohort 2 pre-hierarchy stabilization, (d) Cohort 2 post-hierarchy stabilization.
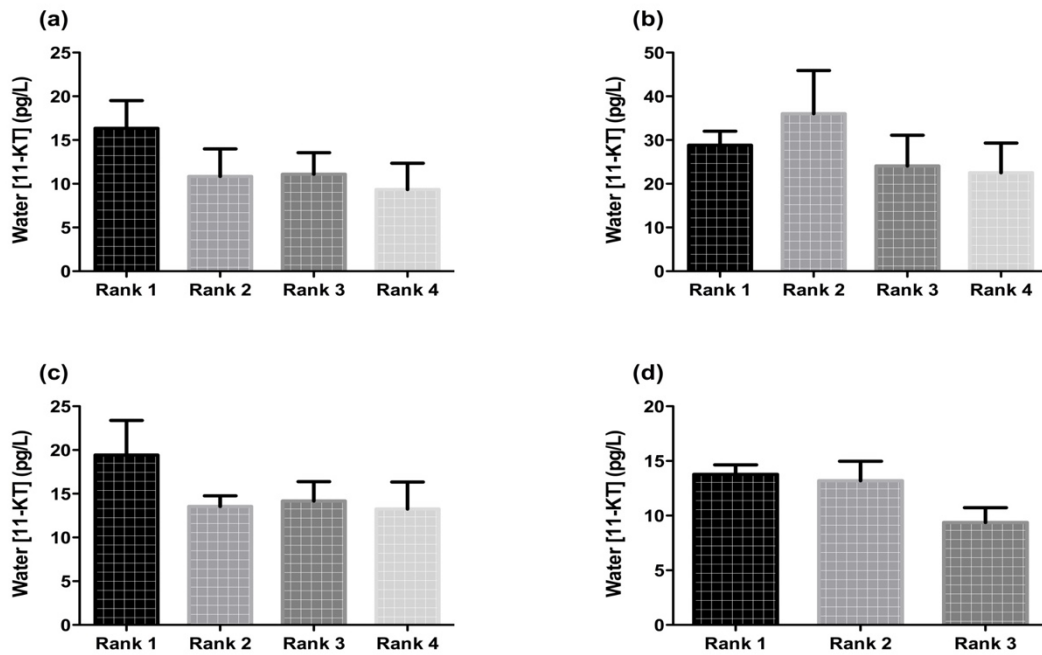
Figure 3.2: Mean ± SE concentration of waterborne 11-ketotestosterone within dominance ranks determined via David's Score dominance index. (a) Cohort 1 pre-hierarchy stabilization, (b) Cohort 1 post-hierarchy stabilization, (c) Cohort 2 pre-hierarchy stabilization, (d) Cohort 2 post-hierarchy stabilization.

Figure 3.3: Mean ± SE concentration of waterborne testosterone within dominance ranks determined via David's Score dominance index. (a) Cohort 1 pre-hierarchy stabilization, (b) Cohort 1 post-hierarchy stabilization, (c) Cohort 2 pre-hierarchy stabilization, (d) Cohort 2 post-hierarchy stabilization.
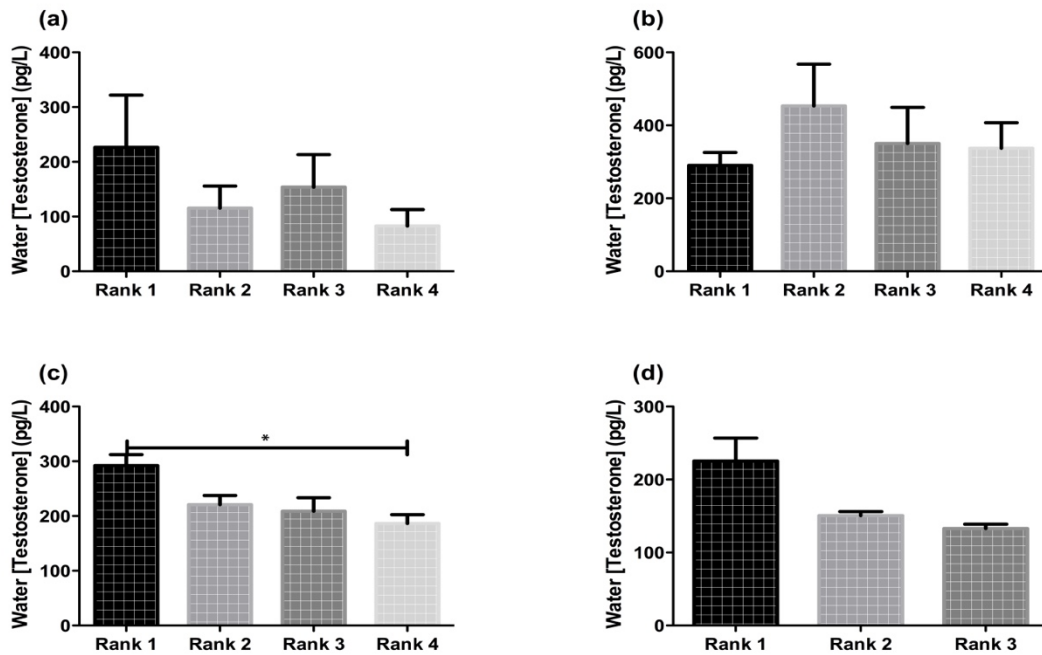
CHAPTER IV: CONCLUSIONS AND FUTURE STUDIES

The current investigation into dominance hierarchy establishment and maintenance in the false percula anemonefish *Amphiprion ocellaris* provides a framework within which to assess hypotheses that suggest that complex animal societies are founded strictly on particular within-group asymmetries (e.g., size variation: Chase 1982; Koebele 1985; Buston and Cant 2006), which in actuality may be consequences of finer-point mechanisms that modulate social status. The current investigation found evidence to suggest that individual aggressiveness is the primary driver of social dominance in novel size-matched groups (chapter 2) and that variation in stress and cortisol expression profiles are related to individual dominance status in both unstable and stable hierarchies (chapter 3). In contrast to contemporary hypotheses suggesting that dominance hierarchies across *Amphiprion* spp. are based strictly on size differences (Buston 2003), my investigation provides evidence demonstrating the integrative impact of behavioural variation and distinctive stress profiles on the modulation of individual social status.

While anemonefish behaviour has been well-studied in recent decades (e.g., Ross 1978; Ochi 1989; Godwin 1994), the number of studies exploring the function and maintenance of anemonefish social structures is fairly limited. Hence, generalizations on the modulation of individual social status have become tenuous and widespread. Across taxa, it is likely that widely-reported size hierarchies occur in concert with established social hierarchies (Forrester 1991;

Koebele 1985; Buston and Cant 2006). However, to suggest that size hierarchies dictate the function of social hierarchies provides an erroneous description that requires remediation via comprehensive study of both behavioural and physiological correlates of social dominance.

In chapter 2, individual aggressiveness was a primary determinant of social dominance. Additionally, dominance rank was not necessarily related to body size. The attribution of high-ranked dominance status to aggressive smaller fish experimentally recruited into established size-stratified groups provided further corroboration for aggression being a critical driver of dominance in *A. ocellaris* groups. Collectively, the findings reported in chapter 2 suggest that *A. ocellaris* hierarchies may not adhere to the previously-reported size-based hierarchy hypothesis (Buston 2003).

While the results reported in chapter 2 provided evidence suggesting that aggression is a critical driver of dominance in *A. ocellaris* groups, it may be beneficial to consider the evaluation of finer-point complexities regulating the dynamics of individual social structures. In previous investigations of social hierarchies, aggression between size-matched individuals has been proposed to act as a means of growth suppression (Nakano 1995). In the long-term observation component of chapter 2, the allocation of dominance to smaller fish invites the question as to whom aggression is directed towards if subordinates are physically larger in stable social contexts. Further, the subordination of larger fish may present an opportunity to consider whether such individuals are actively

avoiding agonistic interactions with social dominants in order to avoid the potential growth-suppressive effects of subordination. If measured alongside growth rate, refined evaluation of within-group interactions may further elucidate the behavioural mechanisms underlying hierarchy formation and modulation of dominance rank in social groups established with an initial absence of a size asymmetry.

The hyper aggressive behaviour (performed by smaller fish of cohort 2) reported in chapter 2 provides impetus to consider the fitness benefits of investing in acute aggression during times of social instability. Given the energetic costs associated with investment in aggressiveness (Marler and Moore 1988; Hofmann and Schildberger 2001; Aureli *et al*. 2002), the practicality of engaging in hyper aggression becomes conceivably dubious. However, when considered in the context of a permanent social aggregation, initial elevated investment may translate into greater fitness benefits. Hence, consideration should be given to the overall fitness benefits associated with ostensibly costly behaviours performed during dominance hierarchy establishment and formation.

In chapter 3, stress profiles were related to dominance status and provided indications of within-group social stability. Additionally, androgen expression was typically highest in top-ranked dominants, albeit that trends reported in chapter 3 were not statistically significant. Collectively, these results corroborate previous reports in which stress and androgen expression profiles provided a representation of within-group social dynamics.

The concurrence of heightened cortisol expression in top-ranked dominants and lowest-ranked subordinates presents an opportune avenue to consider the impact of social stress on the function of energetic metabolism. In other fish species, experimental elevation of cortisol levels typically results in increased mobilization of energy reserves (e.g., Laiz-Carrion *et al*. 2002). In the context of dominance, similarity of stress profiles in dominants and subordinates may be reflective of similar increases in energy expenditure. The stress of dominance has typically been associated with persistent aggressiveness (Creel *et al*. 1996; Fox *et al*. 1997), which in turn requires heightened energy investment (Aureli *et al*. 2002). Conversely, the stress of subordination may be related to elevated energy expenditure associated with escape and submission behaviours (Creel *et al*. 1996; Fox *et al*. 1997; Creel 2001). For both, there are likely long-term implications associated with resource-holding capacity that may impact the dynamics within a social group. Hence, future studies should consider the impact of social stress on energetic metabolism, as well as the overarching fitness consequences that may be associated with the stresses of dominance and subordination.

Collectively, this thesis contributes to our overall understanding of the proximate mechanisms underlying dominance hierarchy formation and maintenance. Individual variation in behaviour and hormone expression profiles reported in chapters 2 and 3 provide a basis from which to consider additional mechanisms that may impact social dynamics within permanent animal aggregations. In general, the reported findings in this thesis indicate that *A.*

ocellaris social organization may be controlled by complex interactions between behavioural variation and distinctive stress profiles. Beyond applications to gregarious marine hermaphroditic fish species, these findings have potential implications towards our overall understanding of the role of aggression and social stress in the modulation of social status in many complex animal societies.

The permanence of anemonefish colonies, coupled with high within-group relatedness, provides an opportune avenue to study the function and evolution of social behaviour in gregarious marine hermaphroditic fishes. Through such investigation, our understanding of the evolution of social behaviour can eventually be extended to a comprehensive understanding of individual variation in behaviour and differences in group structure across taxa. Here, I have shown that individual variation in aggressiveness and hormone expression are related with dominance status within novel and established dominance hierarchies. In future, consideration should be allocated towards fine-point group structure analysis, quantification of energetic metabolism, neuronal variation within-groups, as well as between-group competition that may affect reproductive success and modulation of social status.

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Vita Auctoris

Name: Eugene Cheung

Place of Birth: Hong Kong Special Administrative Region

Year of Birth: 1990

Education: St. Robert Catholic High School, Thornhill, Ontario
                     2004 - 2008, International Baccalaureate

                     University of Toronto, Toronto, Ontario
                     2008 - 2012, H.B.Sc.

                     University of Windsor, Windsor, Ontario
                     2013 - 2015, M.Sc.

                     North Carolina State University, Raleigh, North
                     Carolina
                     2015 – 2020 (expected), PhD.