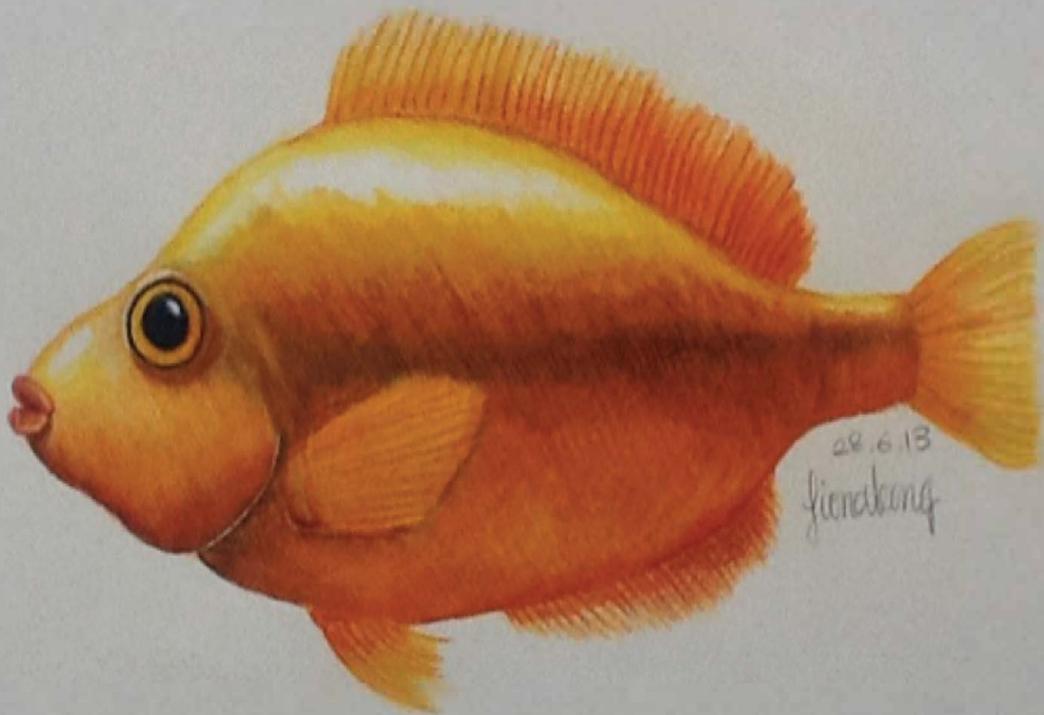


# Behavioural and Morphological Variation in Neotropical Cichlid Fishes



Will Sowersby  
PhD Thesis  
2015

Supervisor: Bob BM Wong  
Co-supervisors: Topi K Lehtonen & David G Chapple



MONASH University

***Behavioural and morphological variation in Neotropical cichlid fishes***

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A thesis submitted for the degree of *Doctor of Philosophy* at  
Monash University in 2015  
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# Abstract

Within species variation provides the building blocks for natural selection to act upon. However, this intraspecific variation represents a puzzle for evolutionary biologists, as natural selection is hypothesised to favour optimal phenotypes and eliminate variation from populations. One large taxonomic group, the cichlid fishes (Cichlidae), is proving to be an unparalleled system for the study of biological variation, population diversification and speciation. In particular, cichlids radiate rapidly, specialise ecologically, can be phenotypically plastic, and often display convergent evolution across habitats. Indeed, closely related species can occur in sympatry, only varying conspicuously in regard to body colour or trophic characteristics, with similar levels of variation even occurring within and between populations. Accordingly, my PhD thesis used Neotropical cichlids as a model to explore the mechanisms underpinning intraspecific variation, and how this variation is both generated and maintained. My thesis is organized into two distinct sections and comprises of four data chapters. In section 1, I investigated how morphological variation, specifically colour polymorphism and trophic morphology, may be generated and maintained in the red devil cichlid, (*Amphilophus labiatus*). In section 2, using both the red devil and another Neotropical cichlid species, the poor man's trophus (*Hypsophrys nematopus*), I investigated key behavioural differences between the sexes in the context of parental investment in the defence of offspring from conspecific and herterospecific territory intruders. Using a multi-disciplinary approach – combining behavioural experiments, genomic analysis and ecological data – I found that within cichlid populations, individuals differ in regard to their trophic morphology, body colour and parental investment and that these differences are directly associated with differences in diet, background matching ability and sex. Specifically, I found significant variation in the phenotypic response of cichlid individuals introduced to a novel environment, clear behavioural and morphological variation between distinct colour morphs, and significant sex differences in the level and timing of parental investment. Together, these results highlight the high level of variation within Neotropical cichlid populations and provide insights into both the generation and maintenance of intraspecific variation.

# Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

William Sowersby 25/11/2015



# Publications during enrolment

## Thesis including published works General Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes four original papers published in peer-reviewed journals (three attached in the appendix) and three unpublished publications. The core theme of the thesis is behavioural and evolutionary ecology. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the School of Biological Sciences under the supervision of Associate Professor Bob B.M. Wong and co-supervision of Dr Topi K. Lehtonen and Dr David G. Chapple. (The inclusion of co-authors reflects the fact that the work came from active collaborations between researchers, and acknowledges input into team-based research.)

In the case of chapter 2, 3, 4 & 5 my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status	Nature and extent (%) of students contribution
2	Morphological variation is correlated with diet in an introduced cichlid fish population with reduced genetic diversity	Submitted manuscript	85%
3	Background matching ability and the maintenance of a colour polymorphism in the red devil cichlid	Published ( <i>Journal of Evolutionary Biology</i> )	95%
4	Temporal and sex-specific patterns of breeding territory defence in a colour polymorphic cichlid fish	Submitted manuscript	95%
5	Heterospecific intruder recognition, mate desertion and sex specific aggression in a biparental cichlid fish	Submitted manuscript	85%

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

**Student signature:** 

**Date:** 25/11/2015

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student and co-authors' contributions to this work.

**Main Supervisor signature:** 

**Date:** 25/11/2015

# Acknowledgements

*This thesis could not have been completed without the support, love, advice and hard work from the following people.*

*My supervisors Bob Wong, Topi Lehtonen and David Chapple, who throughout my PhD have been extremely supportive, offered valuable advice, patience and shared their vast knowledge and experience.*

*My beautiful partner, Johanna Laurent. My family, Tony Sowersby, Carol Hankinson, Nick Sowersby, Bridge Sowersby, Mary and Brian Sowersby, Grace and Norm Hankinson, my extended family and friends.*

*The Wong and Chapple lab groups, in particular, Nicholas Deal, Krystina Mossop, Patrick Tomkins, Marcus Michelangeli, Lynette Plenderleith, Nicholas Moran, Rowan Jacques-Hamilton, Celine Goulet, Ben Wegener, Nori Kawasaki, Rebecca Bray, Anna Senior, Michael Bertram, Mel Klamt, Marie Henriksen, Topi Lehtonen and Minna Saaristo, in addition to several Honours and undergraduate students*

*I wish to thank the following people for their advice, equipment and logistic support, Mark Ravinet and the staff of the DNA Database of Japan (DDBJ), Marta Barluenga and the Museo Nacional de Ciencias Naturales, Walter Salzburger, Bernd Egger and Universität Basel, Christian Beisel and the ETH Zurich Department of Biosystems Science and Engineering in Basel, John Fitzpatrick, Gil Rosenthal, Alysha Heimberg, Kaspar Delhey, Andreas Svensson, Alistair Evans, Devi Stuart-Fox, Ricardo San Martin, Stewart Crawley, Tomas Zohara and Fiona Hibbert.*

*For their help with fieldwork and laboratory work, I thank, Carlos Garita Alvarado, Fiona Kang, Melinda Hofman, Mariana Leal Cardin, Gema Aguilera Garcia, Rowan Jacques-Hamilton, Nicholas Deal, Rachel Fetherston, Eeling Ng, Ruby Albury, Andrej Hohmann, Stefanie Forster, Madeleine De Jong, Hope Calvert, Matthew Simpson and several others.*

*Thank you to the Ministerio del Ambiente y los Recursos Naturales, Nicaragua and the Department of Primary Industries, Victoria, for approving research and collection permits. The Holsworth Wildlife Endowment Fund and the Linnean Society of New South Wales generously provided funding grants.*

*I am very grateful to Monash University and the staff and students of the School of Biological Sciences. Lastly, to my friends and also my support group in the department, Adrian Kirke, Jessie Cosgriff, Lara Devendranath, Ian Laurent, Karen Laurent, Ash Shanahan, Nataly Aranzamendi, Manoj Kamalanathan, Paulina Mikulic, Sean Williamson, Leo Guida, Ly Dao, Thomas Lines, Lotte Van Boheemen, Amanda Peterson, EeLing Ng, Ted Chang, Karin Svanfeldt, Rowan Mott, Stephen Gipson, Susie Ho, Mattia Pierangelini, Yussi Delgado, Melissa Wartman, Marie Fan, Raqal Vaz, Marcelo Lagos, Hannes Eimer, Fariba Pakdel, Nikki Kowalczyk, Derek Dapp, Julie Groce, Niki Teunissen, Huon Clarke, Kylie Soanes, Teresa Mackintosh, Tamsyne Smith-Harding, Angélica Martínez Bauer, Alissa Monk and many others.*

*Cover Art by Fiona Kang.*

*"I've never met an animal, or a plant for that matter, that wasn't interesting, but some stand out as special. Cichlid fishes are right up there. In sheer number of species, they are one of the most successful of all families of vertebrate animals. The extent and speed with which they have evolved in some African lakes has made them the darlings of evolutionary biologists, and the attention is well deserved. That aspect of their biology fascinates me, but what captivates me even more is the complexity of their social lives and their devotion to family, not to mention the sheer beauty of many species."*

George Barlow

The Cichlid Fishes: Nature's Grand Experiment in Evolution

# Behavioural and morphological variation in Neotropical cichlid fishes

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# Chapter One: Introduction

Intraspecific variation provides the raw materials for natural selection to act upon and, is thus, a key focus for understanding how and why organisms diversify (Bolnick et al., 2003, Pfennig et al., 2010, Tills et al., 2011). However, intraspecific variation also represents a puzzle for evolutionary biologists, since natural selection has been hypothesised to favour optimal phenotypes and eliminate all others from a population (Williams 1992). Consequently, a major goal of evolutionary biology has been to understand the selection pressures and mechanisms that drive variation within species (Pfennig et al. 2010). Currently however, the underlying causes of diversification are only understood in a minority of organisms (Kocher 2004). Accordingly, my PhD thesis explores the mechanisms underpinning intraspecific variation and how this variation is maintained within populations.

## ***Ecological specialisation***

In recent decades, there has been a shift away from the more traditional emphasis on geographical isolation in population diversification, with a move towards a broader understanding of how populations diversify (Schluter, 2000, Sorenson et al., 2003, Kocher, 2004, Doebeli et al., 2005, Rundle & Nosil, 2005, Savolainen et al., 2006, Ghalambor et al., 2007, Muschick et al., 2011). Indeed, many studies have now highlighted how specialisation to local environments can also be an important driver of species diversity (so called 'ecological speciation' sensu Schluter, 1996, Schluter, 2000, Sorenson et al., 2003, Savolainen et al., 2006, Schluter, 2009). Specifically, within populations, individuals can show

differential niche use, which can lead to partial reproductive isolation and even the early stages of population divergence (Smith & Skúlason, 1996). Under the ecological speciation hypothesis, resource based divergent natural selection is the ultimate cause of diversification (Huxley 1942). Well-known examples of differential niche use within populations include species of spadefoot toad (*Spea sp.*) whose juveniles can develop into either large cannibalistic morphs or smaller omnivorous morphs (Pfennig, 1999), Minckley's cichlids (*Herichthys minckleyi*) in which individuals differ in regard to their diet and lower pharyngeal jaw structure (Hulsey et al. 2005), and benthic and limnetic populations of sticklebacks (Gasterosteidae) (McKinnon & Rundle, 2002). The formation of these 'ecotype' morphs is often associated with the colonization of novel habitats that are often isolated (e.g. lakes and islands), which reduces the chance of subsequent gene flow into the habitat and also allows for rapid adaptation to vacant niches (Skúlason & Smith 1995, Barluenga et al., 2006, Elmer et al., 2010a). Adaptive radiations can occur, when a lineage diversifies into several species that differ ecologically and have morphological traits that allow them to exploit different resources (Huxley 1942, Futuyma 1986, Schluter 1996). Adaptive radiations are particularly spectacular when they occur rapidly and contain species with a high degree of morphological and ecological differentiation, for example as observed in Galapagos finches (Lack 1947, Grant 1986) and Hawaiian honeycreepers (Amadon 1950). However, despite recent theoretical and empirical evidence providing support for ecological speciation, the role of ecological forces favouring and maintaining reproductive isolation in nature remains poorly understood.

Across their wide range, cichlid fishes (Cichlidae) are ecologically diverse and have also undergone spectacular adaptive radiations (Sturmbauer, 1998). The striking diversity of feeding niches, for example in cichlids inhabiting the East African Rift Lakes (Liem, 1973, Liem, 1980, Kaufman et al., 1997), suggests that niche differentiation occurred by rapid ecological specialisation. In new habitats, the ability to respond plastically to novel food sources can be critical in promoting the diversification of trophic morphology within a population (Wente & Phillips, 2003, West-Eberhard, 2003, Ledon-Rettig et al., 2008, Wund et al., 2008). Furthermore, recent research has revealed that within populations, individuals that differ in the expression of morphological traits and diet may also exhibit genetic differences and even various stages of reproductive isolation (Skúlason et al., 1999, Adams & Huntingford, 2004, Mallet, 2008, Hendry, 2009, Pfennig & McGee, 2010).

Phenotypic plasticity (i.e., the ability of an organism's phenotype to vary in response to its environment) appears to be a major contributor to population diversification (Smith & Skúlason, 1996, Ghalambor et al., 2007, Pfennig et al., 2010), particularly within novel environments (Kocher, 2004, Doebeli et al., 2005, Pfennig & McGee, 2010). Moreover, phenotypic plasticity appears to be taxonomically widespread (Meyer, 1987, Wimberger, 1994, West-Eberhard, 2003, Yeh & Price, 2004, Pfennig et al., 2010) and a rapid source of morphological variation. Nevertheless, the role of phenotypic plasticity in adaptation remains controversial (Pfennig et al., 2010) and has even been viewed as an impediment to evolutionary change (Schlichting & Murren 2004).

### ***Colour polymorphism***

Notwithstanding the considerable theoretical interest, our knowledge of the mechanisms driving intraspecific variation in nature is still relatively rudimentary (Kocher 2004). For example, many closely related species appear to differ only in respect to one or two non-trophic traits (e.g. colouration), suggesting that ecological specialization is not the only mechanism driving intraspecific diversification (Seehausen et al., 1997, Kornfield & Smith, 2000, Baric et al., 2003). Indeed, differences in body colour can occur *within* species and provide a readily observable example of intraspecific variation (Gray & McKinnon, 2007, Williams et al., 2012). However, despite attracting a great deal of research attention, the adaptive and ecological significance of colour polymorphism, its role in population diversification, and the mechanisms that maintain colour frequencies remains controversial (Roulin, 2004, McGraw, 2006, Gray & McKinnon, 2007, Forsman et al., 2008, Wagner et al., 2012, Kusche et al., 2015). This is largely because, in the absence of specific selection pressures, a better performing colour morph should be favoured by selection and drive others to extinction. Moreover, without specific selection pressures, discrete morphs could also be expected to disappear through random events, such as genetic drift (Coyne & Orr, 2004; Rosenblum et al., 2012).

The maintenance of a stable colour polymorphism is likely to be the result of complex interactions, including behavioural and physiological differences between individuals. Indeed, body colour has often evolved in association with behavioural and physiological traits, such as reproductive strategy, aggression,

immune function and stress response (Barlow, 1983, Sinervo & Svensson, 2002, Pryke & Griffith, 2006, Pryke, 2007, Dijkstra et al., 2008, McKinnon & Pierotti, 2010). As such, colour polymorphisms can also be closely linked to behavioural variation. For example, in Gouldian finches (*Erythrura gouldiae*), different colour morphs employ distinct behavioural strategies, particularly in regard to aggression, which is associated with differences in hormone expression and immune performance (Pryke et al., 2007). In other taxa, such as the guppy (*Poecilia reticulata*), predation impacts on the variation and distribution of colour variants, with individuals being less visually conspicuous in areas where predation pressure is higher (Endler, 1980, Young et al., 2011). Colour polymorphisms are therefore ecologically relevant and have even been directly associated with population diversification and sympatric speciation (Wagner et al., 2012).

### ***Sex differences in behaviour and parental investment***

Within populations, behavioural and morphological differences between the sexes can be important. Such differences may often be related to conflict over mating and/or parental investment as each sex seeks to maximize its own reproductive payoffs – even if this occurs at the expense of the other (Parker, 1979, Houston et al., 2005). Providing care for offspring (e.g. guarding young from predators) has obvious fitness benefits. For instance, in the Seychelles warbler (*Acrocephalus sechellensis*), egg loss was found to be seven times higher in unattended nests compared to those that were guarded by parents (Komdeur

& Kats, 1999). Parental behaviour can therefore play a key role in parental fitness and offspring success. However, despite the benefits, agonistic behaviour related to offspring defence can also be costly for parents, in terms of energy loss (Haller, 1996), reduced foraging opportunities (Requena et al., 2012), heightened risk of injury and mortality (Marler & Moore, 1988, Lappin & Husak, 2005), as well as lost future mating opportunities (Trivers, 1972, Székely & Cuthill, 2000).

Consequently, when both parents are required for the successful defence of offspring and breeding territories, parental investment is often not shared equally between the sexes and can become a source of conflict (Trivers, 1972, Wynne-Edwards, 1995). In many species, conflicts can arise, for example, due to differences between the potential reproductive rates of males and females (Baylis, 1981, Reynolds, 1996), or because males may lack assurance over the paternity of the offspring they are raising (Trivers, 1972, Keenleyside, 1991, Neff, 2003). To date, most studies of biparental behaviours have focused on the relative investment of the sexes in the context of offspring provisioning (Harrison et al., 2009), despite the potentially high costs and benefits associated with the aggressive defence of offspring, particularly for reproductive success. Furthermore, surprisingly few studies have taken an experimental approach to investigate how the sexes might alter their investment in parental behaviours over the course of the breeding cycle. Such experimental manipulations are, however, important if we are to gain a more comprehensive understanding of why the sexes vary in their investment in territory defence, especially as this is a key aspect of parental care in many taxa.

## **Neotropical cichlid fishes**

Cichlid fishes provide an unparalleled system for the study of intraspecific variation, biological diversification and speciation (Schilewen, 1994, Kocher, 2004, Barluenga et al., 2006, Seehausen et al., 2008,). Cichlids have shown a propensity to radiate rapidly, specialise ecologically, be phenotypically plastic and display convergent evolution across habitats (Meyer, 1987, Seehausen, 2006, Elmer et al., 2010a, Colombo et al., 2012, Muschick et al., 2012, Santos & Salzburger, 2012). Furthermore, many closely related species frequently occur in sympatry, often only varying conspicuously in regard to body colour or trophic characteristics (Seehausen et al., 1997, Seehausen, 1999, Wagner et al., 2012). Cichlid fishes inhabiting the East African Rift Lakes have speciated faster than any other vertebrate group on earth (Lande, 2001), having undergone an explosive adaptive radiation within a short timescale (Meyer, 1990, Verheyen et al., 2003). More generally, cichlid fishes show an incredible diversity of feeding structures and foraging behaviours, facilitated by a release in the functional constraints, and also the plasticity, of their trophic structures. This has led to extensive ecological diversification and resource polymorphisms, both between and within cichlid species (Skúlason & Smith, 1995).

In comparison to the super-flocks of the East African Lakes, the evolutionary origins of cichlids in Central America (Neotropics) has been the subject of comparatively less research. This is true despite the fact that some lineages have also radiated rapidly (albeit at a smaller scale) and are also highly variable in

trophic morphology and body colour (Barlow & Wallach, 1976, McKaye, 2002, Barluenga & Meyer, 2004, Barluenga & Meyer, 2010, Elmer et al., 2010b).

One particular group of Neotropical cichlids, the Midas species complex (of the genus *Amphilophus*), is proving to be an emerging model system for the study of adaptive speciation, ecological specialisation and phenotypic plasticity (Meyer, 1987, Barluenga et al., 2006, Muschick et al., 2011). Within the Midas complex, variation between species often only consists of one or two key traits, which may also occur within species, making it likely that this group can offer additional and perhaps unique insights into how variation in species is generated and maintained, particularly in novel habitats.

## **Aims**

Using Neotropical cichlids as a model, my PhD thesis explores mechanisms underpinning intraspecific variation, and how this variation might be maintained. My thesis is organized into two sections, comprising four data chapters. In section 1, I investigated how morphological variation may be generated and maintained in the polymorphic red devil cichlid, *Amphilophus labiatus*. In section 2, using red devils and another Neotropical species, the poor man's tropheus, *Hypsophrys nematopus*, I investigated key behavioural differences between the sexes in the context of parental investment in offspring defence. Together, the results of my thesis provide insights into our understanding of intraspecific variation in key morphological and behavioural traits. Apart from my own PhD work, over the course of my candidature, I also participated in several additional studies investigating aggression, intruder

recognition, parental behaviours and territory defence in Neotropical crater-lake cichlids. The papers arising from these studies are attached to the Appendix of my thesis.

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# SECTION ONE

## Declaration for Thesis Chapter

Monash University

## Declaration for Thesis Chapter Two

### Declaration by candidate

In the case of Chapter Two, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conceived and designed study, Conducted fieldwork and laboratory work, Analysed data, Aided with bioinformatic analysis, Wrote chapter/manuscript	85%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

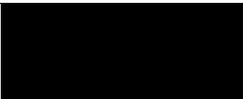
Name	Nature of contribution	Extent of contribution (%) for student co-authors only
<b>Bob Wong</b>	Helped design study, aided with fieldwork, proof read and contributed to manuscript	
<b>Topi Lehtonen</b>	Helped design study, aided with fieldwork and analysis, proof read and contributed to manuscript	
<b>Mark Ravinet</b>	Assisted with analysis, proof read manuscript	
<b>Marta Barluenga</b>	Aided with some fieldwork and provided laboratory equipment	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's  
Signature

	Date 25/11/2015
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Main  
Supervisor's  
Signature

	Date 25/11/2015
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# Chapter Two: Morphological variation is correlated with diet in an introduced cichlid fish population with reduced genetic diversity

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**Submitted manuscript**



*A red devil cichlid with large hypertrophied lips, collected from Lake Nicaragua, Nicaragua.*

## Abstract

Ecological specialisation plays a major role in the evolution of phenotypic diversity, especially following the colonization of novel habitats. In this respect, cichlid fishes provide some of the most remarkable examples of rapid ecological diversification. Here, we capitalized on a recently (~40 years) introduced population of red devil cichlids in South-eastern Australia to investigate phenotypic responses to novel environmental conditions. Specifically, we used stomach content analyses, laboratory experiments and restriction site associated (RAD) sequencing to test associations between morphological variation and diet. We found that the proportion of both algae and macroinvertebrates in the diet were correlated with lip size in the introduced population, despite their maximum lip size being smaller than in the natural range. However, we did not find evidence for lip development in juveniles to be plastic in relation to substrate complexity or diet manipulation, nor was there a significant relationship between lip morphology and feeding performance in adults. Single nucleotide polymorphism (SNP) data, in turn, suggest that a population bottleneck has reduced standing genetic variation, in the introduced population, potentially influencing both phenotypic plasticity and diversity compared to natural populations. Together, the results suggest that, despite reduced genetic diversity following a recent introduction, morphological variation in a key trophic trait can be closely linked to exploitation of novel resources.

**Keywords:** ecological specialization, introduced species, Midas cichlid, phenotypic diversity, plasticity, RAD tag sequencing

## Introduction

Ecological specialisation to local environments is now accepted as a major driver of phenotypic diversity, both between and within species (Schluter 2000; Sorenson et al. 2003; Savolainen et al. 2006; Muschick et al. 2011). For example, intraspecific morphs that show differential niche use (i.e. segregation in habitat and diet) have been linked to the intermediate stages of population divergence (Smith & Skúlason, 1996). Well-known examples of this phenomenon include benthic and limnetic populations of sticklebacks (McKinnon and Rundle 2002) and trophic specialization in whitefish (Lu and Bernatchez 1999). Such examples are often associated with the colonization of novel habitats that are often isolated (e.g. lakes and islands) and thus allow rapid adaptation to empty niches. However, despite the potentially rapid nature of these events, their beginnings are rarely witnessed in the wild, and the underlying processes and mechanisms that facilitate ecological diversification are still not completely understood.

In new habitats, the ability to respond plastically to novel food sources can be critical in promoting the divergence of trophic morphology, potentially generating selection for extended phenotypic responses (Wente and Phillips 2003; West-Eberhard 2003; Ledon-Rettig et al. 2008; Wund et al. 2008). In this respect, adaptive phenotypic plasticity (i.e., the ability of an organism's phenotype to vary in response to its environment) appears to be a major contributor to population diversification (Smith and Skúlason 1996; Ghalambor et al. 2007; Pfennig et al. 2010), particularly within novel environments (Kocher 2004; Doebeli et al. 2005; Pfennig and McGee 2010). Nevertheless, the role of phenotypic plasticity in adaptation has been controversial (Pfennig et al. 2010)

and often viewed as an impediment to evolutionary change (reviewed in Schlichting 2004), despite being taxonomically widespread (Meyer 1987; Wimberger 1994; West-Eberhard 2003; Yeh and Price 2004; Pfennig et al. 2010).

One important case of phenotypic plasticity in diversification is the explosive adaptive radiations of cichlid fishes (Skúlason et al., 1999, Adams & Huntingford, 2004, Mallet, 2008, Hendry, 2009, Pfennig & McGee, 2010). An interesting feature of adaptive radiations, including those of cichlid fish, is that species occupying similar niches often independently evolve similarities in particular traits (West-Eberhard, 1989, Galis & Metz, 1998, Stauffer & van Snik Gray, 2004, Clabaut et al., 2007, Muschick et al., 2011). A well-known example of this convergent evolution is the development of large hypertrophied lips, which are often associated with an elongated head (e.g., Klingenberg et al. 2003). This feature has evolved repeatedly in different cichlid lineages in East Africa (Kocher et al. 2003; Arnegard et al. 2001; Salzburger et al. 2005; Oliver and Arnegard 2010; Colombo et al. 2012) and the New World (Klingenberg et al. 2003; Elmer et al. 2010b, 2010c; Colombo et al. 2012; reviewed in Burrell 2015).

It has been hypothesized that large lips could facilitate feeding on invertebrates, in particular crustaceans, from crevices and gaps between rocks (Barlow and Munsey 1976; Seehausen 1996; Konings 1998; Elmer et al., 2010a, Elmer et al., 2010c, Colombo et al., 2012, reviewed in Burrell, 2015). However, this remains largely untested (Manousaki et al. 2013, but see Baumgarten et al. 2015), and alternative hypotheses exist (Fryer 1959; Ferry et al. 2012; Yamaoka 1997; Arnegard et al. 2001). Greenwood (1974) for example, suggested that large lips

could be an adaptation to reduce the mechanical shock of striking rocks during foraging. However, few studies have tried to experimentally disentangle among these hypotheses. There is also limited evidence to suggest that phenotypic plasticity may play a role in the development of lip size. In particular, in captivity large hypertrophied lips reduce in size (Barlow & Munsey, 1976), indicating that there may be a plastic component to the thick lip trait. Interestingly, large-lipped fish often coexist with smaller-lipped forms, with the two types tending to otherwise only differ in regard to their ecological niches (Colombo et al. 2012; Baumgarten et al. 2015). Moreover large-lipped forms appear to have rapidly evolved from small-lipped populations (Elmer et al. 2010a).

The red devil (*Amphilophus labiatus*, Günther, 1864) is an omnivorous species of cichlid fish with large hypertrophied lips. The red devil is a member of the Midas cichlid complex, a group of Neotropical cichlids that has diversified rapidly, particularly after colonizing new environments (Barlow and Munsey 1976; Elmer et al. 2010a, 2010c). In its native range in Nicaragua, there is little or no neutral genetic differentiation between the red devil and its more common small-lipped congener, the Midas cichlid (*Amphilophus citrinellus*) (Barluenga and Meyer 2004, 2010) however, hybrid or intermediate lip phenotypes are rare or absent. In recent decades, a feral population of red devil cichlids has become established in the man-made Hazelwood pondage in south-eastern Australia (NIWA 2008), which, despite being located in a temperate climate, is artificially heated by runoff water from a closeby power station. This introduction into an isolated lake is analogous to a founder population naturally colonising a novel environment (sense Elmer et al. 2010a). For example, in both instances,

individuals may need to adjust foraging behaviors and resource use traits in response to a change in both the available sources and type of food. Hence, the feral red devil population provides an unprecedented opportunity for studying the role of plasticity and resource polymorphism in facilitating ecological diversification after a recent colonization event.

Using a multidisciplinary approach combining laboratory-based experiments, field-based observations, morphological assays and genomic analyses, we set out to investigate whether observed variation in morphology among individuals of the introduced red devil population is associated with differentiation in ecological niche. First, we experimentally investigated whether among individual morphological variation in the introduced population reflected differences in feeding performance in regard to different diet and substrate complexity. Second, focusing on laboratory-reared juveniles from the same population, we aimed to test the extent to which developmental plasticity can shape key trophic traits, in particular, lip size and head shape. Lastly, using genomic data we examined genetic diversity to test whether there was evidence of a bottleneck in the feral population, putatively limiting standing genetic variation, which, in turn, might influence phenotypic diversity and plastic responses.

## Methods

### ***1) Relating morphology and diet in the introduced red devil population***

To investigate whether variation in morphology is correlated with differences in diet in the introduced population, we first collected adult red devils from Hazelwood Pondage in late 2011 and again in early 2012 using bait traps and hand-lines. We euthanized fish with a lethal dose of clove oil (100ml clove oil/ethanol solution per 1L of water) and then dissected and removed the entire stomach and gut of 60 individuals. We separated food items into one or more of the following categories and determined their volume (in %): mollusks, crustaceans, insects, zooplankton, fish (remains, scales and eggs), plant material, algae and other (e.g. silt). Lip size (standardized for body size), in turn, was assessed by analysing a digital photo (Nikon D5200 Digital SLR) taken of the right lateral side of each individual and a 1-mm grid paper as a scale. Using IMAGEJ version 1.47 (Rasband 1997, NIH), we measured the area of the lip, which – when compared to the area of the whole body (excluding fins) – allowed us to calculate lip area as a percentage of body size.

To investigate the relationship between diet and standardized lip size, we applied a single generalized mixed model (GMM). Specifically, gut content (percentage volume) was used as the response variable, with the standardized lip size and food item as fixed effects and individual fish ID as a random effect. Initially, we used a binomial distribution, appropriate for a proportional response variable. However, due to overdispersion of the data (Zuur et al. 2013),

we translated the model to a log-normal-Poisson distribution by including an individual-level random variable (Elston et al. 2001; Lehtonen and Kvarnemo 2015). The full model was then simplified by stepwise removal of non-significant interaction terms (with  $\alpha = 0.05$ ; Crawley 2007).

## **2) Feeding performance experiment**

We carried out a controlled laboratory experiment to determine whether variation in morphology (lip size and head length) is linked to differences in feeding performance in relation to different diet and substrate type.

For this purpose, we collected sub-adults ( $n = 88$ , standard length 60 -75 mm), identified as sub-adults by standard body length (<12 mm), from Hazelwood Pondage in early 2013 using the same methods as described above. Fish were transported back to the laboratory, and were then sorted based on lip size and head length. Standardized lip size was assessed from photographs, as detailed above. In addition, we assessed the standardized head length of each individual, by comparing the absolute head length (distance between the front tip of the lips and the top of the pectoral fin) with standard body length. We found that lip size and head shape were linked, with individuals with thicker lips also having a more elongated head or being thinner lipped with a shorter head (Fig. 2). Each individual was tagged for identification in the caudal peduncle with a visible implant elastomer (Willis and Babcock 1998). Finally, all individuals were also weighed ( $\pm 0.01$  g), in a container of water on an electronic balance.

We found that average lip size was 1.2% of standard body area. To ensure we had a representative sample of lip sizes in our experimental tanks we categorised individuals with lip size above 1.2% as 'large and below 1.2% as 'small lipped. The two groups of fish (i.e. large lips/long heads versus small lips/short heads) were then split approximately equally between eight 250L tanks maintained at 25°C on a 12:12 day night cycle (stocking density = 11 fish of unknown sex per tank). Using a 2 x 2 factorial design, we tested the effects of substrate (fine grain sand versus cobbles and coarse river rocks) and diet type (thawed frozen brine shrimp versus Otohime EP3 fish food pellets) on the feeding performance of the fish (2 tanks per treatment combination). We purposely selected two substrate types that differed in particle size and, consequently, also differed in the size of interstitial spaces and depth of crevices formed between substrate particles. We did this because different lip phenotypes and foraging techniques may be more successful at extracting food from substrate surfaces or from in-between substrate particles (see Baumgarten et al. 2015). The particle size of the fine grained sand was <1.5 mm in diameter, compared to cobbles, which had a diameter of 5-20 mm, and coarse river rocks, which ranged in diameter from 55 to 150 mm. In the native range, the diet of the red devil cichlid primarily consists of hard shelled invertebrates and small arthropods, however they will also eat plant material, seeds, fish and fish scales (Colombo et al. 2012). We therefore selected different diet types to reflect the red devils naturally varied diet. Fish were kept under experimental conditions and fed 5 days a week for eight weeks. Food (30 grams) was placed into each aquarium in a container that was pulled along the bottom of the tank with the aid of fishing line and lead weights so that the food was evenly distributed on the

substrate before fish could start eating. Fish were weighed and photographed each week. All fish survived the eight-week experiment and weight gain during this time period was used as an indicator of feeding performance.

To assess the influence of substrate and diet on the feeding performance (weight gain) of individuals with differing head length and lip size, we fitted a general linear mixed model, using the 'glme' package. The initial full model was fitted with diet, substrate type, and relative lip size (as a continuous variable) as explanatory fixed factors. The response variable, weight gain was square root transformed to meet the assumption of normality of data. To account for the design of the experiment and the potential interdependence between individuals in a treatment tank, tank ID was added as a random effect. The full model was simplified by stepwise removal of non-significant interaction terms (Crawley 2007), using log likelihood ratio tests (with  $\alpha = 0.05$ ). Lastly, we calculated the variation in weight gain/loss in each experimental tank.

### ***3) Experiment on developmental plasticity of trophic traits***

In a separate, longitudinal experiment, we also investigated the extent to which developmental plasticity influences lip size and head shape under different diets and substrate complexity.

Sub-adults collected at the same time and using the same methods as those described previously, were brought back into the laboratory where they were

housed in large tanks (4000 L) maintained at 25°C on a 12:12 day night cycle (stocking density = ~50 fish per tank). Once fish had reached sexual maturity (~5 months after collection), pairs were allowed to form naturally in smaller (1500 L) tanks. During the course of the study, we were successful in obtaining spawnings from three pairs, resulting in three separate clutches. After fry had been free-swimming for 14 days, 96 were collected and removed from the parents for use in the experiment.

To assess the role that substrate complexity and diet plays in influencing the development of lip and head morphology, while controlling for differences between families, we used a 2 x 2 factorial split-clutch experimental design. Specifically, for each clutch, the siblings were split equally across 4 (200 L) same sibling tanks, representing the 4 possible treatment combinations of diet (soft versus hard pellets) and substrate type (sand versus coarse gravel and cobbles; i.e. 12 tanks in total). For this experiment, we manipulated the hardness of the food by soaking the soft pellets in water for 10 minutes prior to the fish being fed. Otherwise, the method for delivering the food was the same as that described in experiment 1. For the first 3 months of their life, fish subsisted on pellets that were 1.7mm in diameter, before progressing onto the 3.1mm pellets for the rest of the experimental period. The entire experiment lasted 14 months, after which, the fish were photographed (left lateral side).

We compared the lip size and head length of each individual ( $n=96$ . Table 1) in IMAGEJ using the same methods as described above. In addition, we quantified the width of the head of each individual by measuring the distance down dorsal-

ventrally from the anterior edge of the dorsal fin.

To assess the influence of substrate and diet on the developmental plasticity, we fitted three separate general linear mixed models, using the package 'glme'. In particular, we had standardized lip size at the end of the experiment, standardized head length, and standardized head width as the response variable in the first, second and third model, respectively. In all cases the food hardness treatment and substrate type were fitted as explanatory fixed factors and, to account for the design of the experiment and the potential interdependence between siblings, clutch ID as a random effect. Each full model was then simplified by stepwise removal of non-significant interaction terms (Crawley, 2007), using log likelihood ratio tests (with  $\alpha = 0.05$ ).

#### ***4) Morphological comparisons with fish from native range***

We quantified and compared the morphology of wild caught red devil cichlids from their native range in Nicaragua and compared these with our feral, Australian population. Red devils were collected from multiple locations in Lake Nicaragua in late 2013 (see supplementary material) and in Australia using the same methods as outlined above. Fish were individually photographed (left lateral side) for morphological analyses ( $n=81$ , from both areas, Table 2).

Using the same methods above, we measured variation in lip size and head shape in native and introduced fish (see supplementary material). We used a Shapiro–Wilk normality test to assess the distribution of lip size in the introduced

population and native Nicaraguan populations. We used R 3.0.0 software for all statistical analyses (R Development Core Team).

### **5) Genetic diversity**

Differences in the extent of plasticity and phenotypic variation in the introduced vs. natural populations may be due to reducing genetic diversity as a result of a population bottleneck during introduction. To test this possibility, we compared the level of genetic diversity in the introduced population with individuals from the native range using RAD-sequencing, a reduced representation population genomic method. We took samples and extracted DNA (DNeasy Blood & Tissue Kit, QIAGEN) from individual samples, from the introduced population and Lake Nicaragua. Samples were digested using the Sbf1 enzyme following a standard RAD-sequencing protocol. We pooled 40 (barcoded) individuals into 5 libraries (total n = 200). Each library was sequenced (single-end) on separate Illumina HiSeq2500 lanes (200 bp reads) at the ETH Zurich Department of Biosystems Science and Engineering in Basel, Switzerland.

Since no reference genome is available for *Amphilophus* spp., we used a *de novo* assembly approach implemented in Stacks v to identify RAD loci and estimate genome-wide diversity (Catchen 2011, 2013). To ensure only high quality reads were used in our analysis, we first trimmed sequenced reads to 175 bp and removed any with an average Phred quality score <20. Quality screening and sample demultiplexing was performed using the Stacks *process radtags* module.

RAD loci were first identified within individuals using the *ustacks* module, allowing a minimum of 5 reads per stack, a maximum of 2 stacks per locus and up to 4 nucleotide mismatches within and between stacks. SNPs were called at the individual level using the standard Stacks SNP model with  $\alpha = 0.01$ .

Following this step, we then used a subset of 50 individuals with the highest read depths from across the native and introduced range to create a *de novo* RAD loci catalogue. In short, this catalogue acts as a database of identified loci amongst all individuals. Care was taken to ensure individuals of both sexes were included to prevent bias. Catalogue construction was performed using *cstacks* with a maximum of 4 mismatches permitted between loci amongst individuals.

Once our *de novo* reference catalogue was complete, the remaining 150 individuals were matched against it using *sstacks* and we used the *populations* module to perform final dataset filtering and to estimate population genetic statistics. Polymorphic RAD loci were only included if they occurred in at least 50% individuals in each of the six populations, had a minimum read depth of 20x and had a minor allele frequency  $> 0.1$ . From our final dataset, we tested for differences in observed heterozygosity and nucleotide diversity amongst the populations using GLMs in R (R Development Core Team).

## Results

### **1) Relating morphology and diet in the introduced red devil population**

The most abundant food items in the introduced population were plant material (23%), algae (22%) and macroinvertebrates (14%; see Fig. 2A).

When we applied the full linear model (gut content or food type with lip size) to assess the effects of food type and relative lip size on gut content, we found a significant interaction between lip size and food item ( $z=-1.988, p= 0.047$ ). Specifically, we found that as relative lip size increased, the proportion of algae in the gut increased, and the proportion of insects decreased (Fig. 2B).

### **2) Feeding performance experiment**

In regard to the feeding performance of wild-caught fish (from the introduced population) under laboratory conditions, after simplification of the full model (i.e. stepwise removal of non-significant interactions), we found a significant main effect of diet ( $z= -4.19, p <0.0001$ ), with individuals consuming pellets gaining more weight. We did not find a significant effect of substrate complexity ( $z= 0.01, P = 0.99$ ) or standardized lip size ( $z= -0.07, P = 0.94$ ) on the amount of weight gained. When we assessed the degree of variation between our treatment tanks in respect to weight gain, we found that differences in variation were associated with diet treatment (supplementary material, table 1).

### **3) Developmental plasticity of trophic traits**

When we assessed developmental plasticity of lip size by applying a generalized mixed model to assess the effects of the substrate complexity and food hardness, the stepwise removal of non-significant factors (i.e. stepwise removal of non-significant interactions) did not reveal any significant interactions or effect of substrate ( $z= -0.166, p= 0.87$ ) or diet ( $z=0.245, p=0.81$ ) on lip development. In our second model, to assess the effects of the substrate complexity and food

hardness on the developmental plasticity of head length, the stepwise removal of non-significant factors did similarly not reveal any significant interactions or effect of substrate ( $z=0.10$ ,  $p= 0.92$ ) or diet ( $z= 0.86$ ,  $p= 0.39$ ). Lastly in our third model assessing the developmental plasticity of head width, the stepwise removal of non-significant factors did not reveal any significant interactions or effect of substrate ( $z= -0.22$ ,  $p= 0.82$ ) or food hardness ( $z= 0.80$ ,  $p= 0.42$ ). Hence, we did not find any evidence for phenotypic plasticity in lip size or head shape during development, in response to either substrate complexity or hardness of the food.

#### **4) Morphological comparison with fish from the native range**

We found that lip size relative to body size was larger in the Nicaraguan population (mean $\pm$   $\sigma^2$  1.81, 0.17) compared to the introduced Australian population (mean $\pm$   $\sigma^2$  1.21, 0.95) (For comparison see Fig 1). Furthermore, we found that lip size is normally distributed in both the native population (Shapiro–Wilk Normality Test,  $W= 0.96$ ,  $P=0.94$ ) and in the feral population ( $W = 0.97$ ,  $P = 0.28$ ). Mean standard body length in Nicaragua was 16.9 cm (range 11.4 - 22.2 cm) and 11.7 cm (range 7.2 – 20.1 cm) in Australia.

#### **5) Genetic Diversity**

Our *de novo* assembly identified 341 226 unique RAD tags, reduced to 6226 RAD loci and 8038 SNPs following filtering for RAD loci which were polymorphic, occurring in all six populations in >50% individuals, had a MAF of >0.1 and were sequenced to at least 20x depth. In the final dataset, an average of 1.29 SNPs occurred on each RAD tag.

We found mean observed heterozygosity was 38% lower in the introduced population compared to populations from the native range (Fig 3; GLM,  $R^2 = 0.05$ ,  $F = 529.5$ ,  $df = 5, 48222$ ,  $p < 0.0001$ ). Similarly, nucleotide diversity was reduced by 34% in the introduced population relative to the native range ( $R^2 = 0.07$ ,  $F = 775.9$ ,  $df = 5, 48222$ ,  $p < 0.0001$ ).

## Discussion

We found a significant correlation between lip size and diet in the introduced red devil population. Specifically, as lip size increased, so did the proportion of algae in the diet; the proportion of insects decreased. Experimentally, we found no significant link between phenotypic variation and feeding performance, when foraging on different substrates or having different diets. Similarly, there did not appear to be any plastic developmental response in lip size or head shape to diet or substrate treatments. Furthermore, compared to individuals in the native range, the lip size of the introduced red devils is smaller and the latter population has significantly lower genetic diversity.

We found that variation in lip size was correlated to differences in diet in the introduced population. Specifically, a greater proportion of algae was observed in stomach and gut content as lip size increased, and a greater proportion of insects as lip size decreased. This suggest that variation in trophic morphology may be linked to the exploitation of different resources. Such a pattern may eventually facilitate a population divergence in respect to ecological niche, as has been observed in the cichlid, *Herichthys minckleyi*, where populations consist of

individuals that exhibit one of two discrete pharyngeal jaw morphologies (Hulseley et al. 2005). Interestingly, despite the high degree of trophic differentiation between *H. minckleyi* ecotypes, there is no evidence of genetic differentiation (Kornfield & Koehn 1975). The exploitation of vacant ecological niches is, however, a primary hypothesis explaining the rapid diversification of Midas cichlids in the Neotropics (Barluenga et al. 2006). For example, Elmer et al. (2010a) found that a large-lipped *Amphilophus* phenotype has evolved from a small-lipped form within historical times (~100 years) in crater Lake Apoyeque, Nicaragua. Interestingly, Lake Apoyeque fish that differed in regard to lip size also differed in ecological niche use, with the thick lipped form consuming more insects and the thin lipped form significantly more algae (Elmer et al. 2010a). By contrast, we found the opposite pattern in the Australian feral red devil population. Why?

In several fish species, large hypertrophied lips appear to be an adaptation for foraging on rocky surfaces, in particular for invertebrates (Colombo et al. 2012; Baumgarten et al. 2015). Indeed, crustaceans and hard shelled invertebrates form the majority of the diet of red devils in Lake Nicaragua and Lake Managua (Colombo et al. 2012). However, in at least some species, large lips seem to have evolved for other functions, and across different taxa (or populations), may be used to exploit several different resources (Agrawal and Mittal 1991). For example, in some African cichlids, large lips have a high density of taste buds and may be acting as an accessory gustatory organ (Arnegard and Snoeks 2001; Oliver and Arnegard 2010). Large lips may also be an adaptation for reducing the mechanical shock of striking rocks during foraging (Greenwood 1974).

Furthermore, large hypertrophied lips appear to have evolved from a large range of ancestral states in cichlids, including, the algivorous *Lobochilotes* (Wagner et al. 2009; Muschick et al. 2012), omnivorous *Amphilophus* (Barluenga et al. 2006; Colombo et al. 2012), and the piscivorous *Crenicichla* (Burress et al. 2013). In the introduced red devil population, large lips may reduce the mechanical shock of foraging on rocky surfaces, or, considering the gut content of large-lipped individuals, be employed to remove algae from benthic surfaces, as in the kissing gourami fish (*Helostoma temminckii*) (Ferry et al. 2012). Apart from algae, crustaceans are an important part of the diet of red devils in their native range (Colombo et al. 2012). The absence of this food source in the introduced habitat may therefore have contributed to the smaller lip size and the use of lips for processing alternative food sources.

Variation in lip size and head shape did not influence feeding performance under different substrate complexity or diet treatments. We did, however, find a significant effect of diet type on feeding performance (weight gain), which was not associated with morphological variation and, instead, is most likely due to nutritional differences between the two food types. In contrast to our study, Baumgarten et al. (2015) found that *Haplochromis chilotes*, a large-lipped cichlid, is more efficient at extracting food from crevices, when compared to the small-lipped congener, *Haplochromis nyererei*. We did not detect a link between feeding performance and morphology in the laboratory, despite the correlation between lip size and diet in the Australian feral population. Therefore, it remains possible that our experiment tested environmental conditions other than those

that may have resulted in the observed relationship between phenotypic variation and feeding in the introduced population. For example, in the introduced population, larger lips may be employed for removing algae from rocky surfaces rather than for drawing food items from gaps and crevices between rocks. In the laboratory, the growth of epiphytic algae on gravel and pebbles was very limited, especially when compared to the availability of the offered food sources.

Our laboratory-based experiments did not provide any evidence for developmental plasticity in lip size or head shape in response to the assessed environmental conditions. In contrast, other studies on cichlids have found that variation in trophic traits can arise from differences in feeding regime (Witte et al. 1989; Stauffer and van Snik Gray 2004; Muschick et al. 2011). For example, Meyer (1987) found the head/snout shape of *Parachromis managuense* siblings differed when they were raised under diet treatments that required different modes of feeding. Our study raised individuals from only three separate clutches and it is therefore possible that a limitation in the number of clutches, individuals or experimental tanks may have contributed to our study not being able to detect any plastic response in lip size to different diet and/or substrate treatments. Furthermore, in red devils, the mechanisms underlying the development of large hypertrophied lips are likely to be complex and their development might require more than exposure to the two particular environmental conditions assessed in this study. In this respect, the reduction in lip size that occurs in red devils held under captive conditions implies a degree of plasticity in this trait (Barlow and Munsey 1976). On the other hand, the

hybrid offspring of captive red devils and smaller-lipped Midas cichlids exhibit an intermediate lip phenotype, suggesting that this trait is also, at least partially, heritable (Machado-Schiaffino et al. 2014).

The introduced red devils had a much lower genetic diversity than populations from the native range, which is a common feature of introduced populations (Barrett et al. 1991; Kinziger et al. 2011) and populations that have recently colonized new environments following range expansions (Bernatchez and Wilson 1998). Standing genetic variation is important for adaptive divergence and the rapid evolution of phenotypically similar traits amongst independent populations (Barrett and Schluter 2008, Schluter and Conte 2009). Population bottlenecks and founder events may reduce variation at adaptive loci segregating in a population, potentially reducing phenotypic diversity and the availability of phenotypic variance for selection to act upon (Barrett and Schluter 2008). Similarly, a reduction in genetic variation may also result in a plasticity response. Increased genetic variation has been hypothesised to increase the potential for phenotypic plasticity in introduced species, as plasticity itself is often a heritable trait (Scheiner 1993; Pigliucci 2005). Therefore, an avenue for further study would be to explicitly test for phenotypic plasticity in a native wild population, in which the current results show genetic variation to be much higher than our introduced population.

In summary, we found that an introduced red devil population is phenotypically variable in regard to lip size. Furthermore, we found that this morphological variation is associated with differences in ecology, specifically diet. This

association may be an initial sign of diversification within the population, with variation in a key trophic trait, hypertrophied lips, being used to exploit different resources. Under experimental conditions, we did not find morphological differences to be associated with feeding performance in the ecological conditions we assessed, nor did we observe any plasticity in these traits during development. Hence, in the introduced population, ecological factors other than those commonly acknowledged (and tested by us) seem to have been driving the diet specialization by red devils with different morphologies. Notably, such morphology-dependent specialization has taken place despite reduced standing genetic variation in the population.

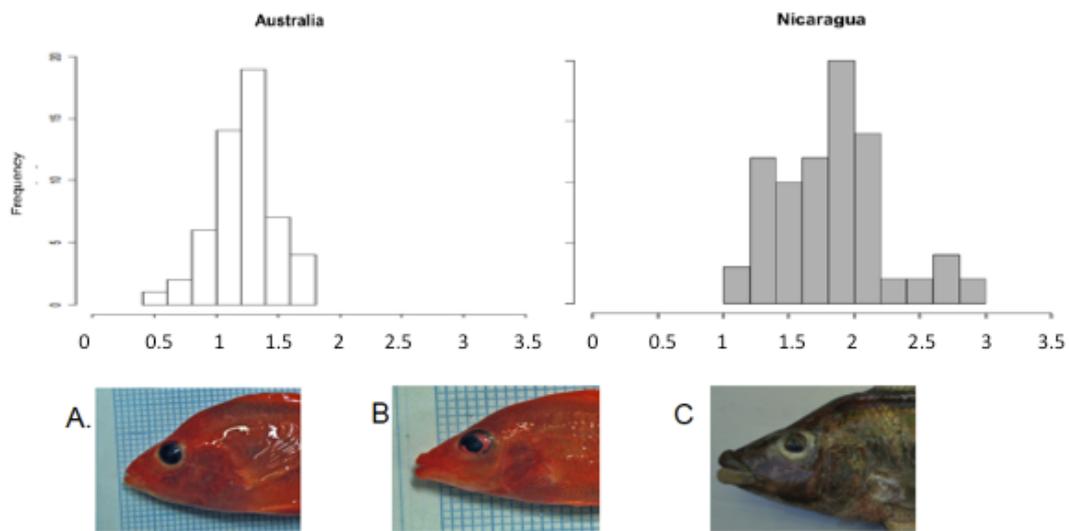
## Tables and Figures

**Table 1** Sample sizes of each substrate/food hardness treatment type.

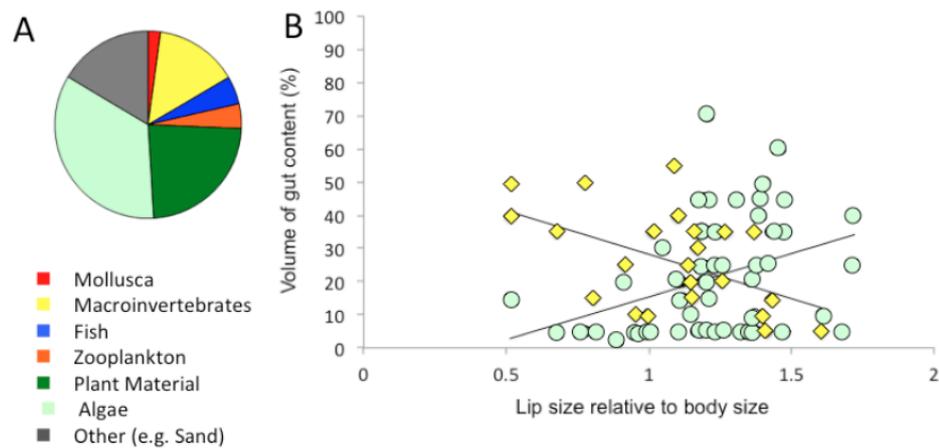
	Soft Food	Hard Food
Sand Substrate	17	31
Rock Substrate	25	19

**Table 2** The number of individuals analysed for phenotypic comparisons between the introduced Australian population (AUS) and native Nicaraguan populations in Lake Nicaragua (LN) and Lake Managua (LM).

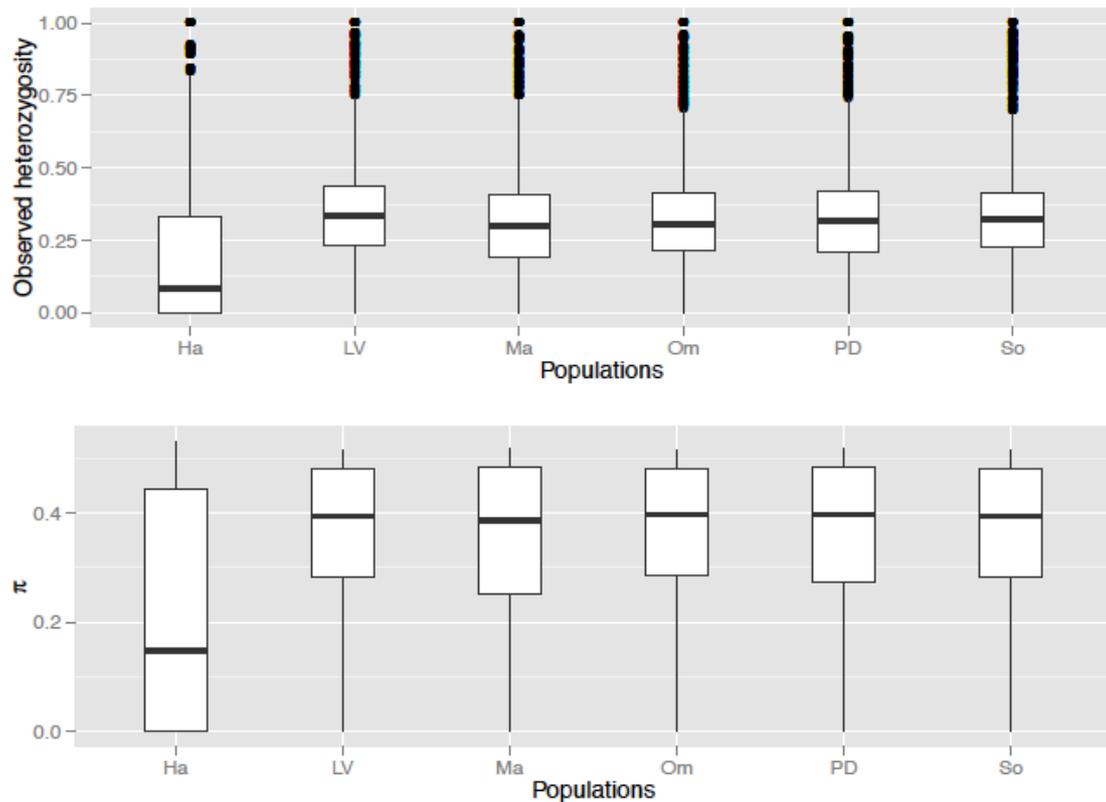
Location	Sample size
Hazelwood Pondage (AUS)	81
La Virgen (LN)	18
Ometepe (LN)	19
Isletas de Granada (LN)	15
Solentiname (LN)	5
Puerto Diaz (LN)	19
Managua (LM)	5

**Figure 1**

**Fig 1.** The distribution of lip size (relative to body size) in the introduced Australian population (left), and from the native population in Nicaragua (right). **A)** Thin lipped, and **B)** thick lipped individual from the introduced population, **C)** an individual from the native range.

**Figure 2**

**Fig 2. A)** Proportion of food items found in the gut and stomach content of red devil cichlids from the introduced population. **B)** The percentage of algae (light green dots) found in the gut and stomachs of red devils increased with lip size (in relation to body size), while the percentage of insects (yellow diamonds) increased as lip size decreased.

**Figure 3**

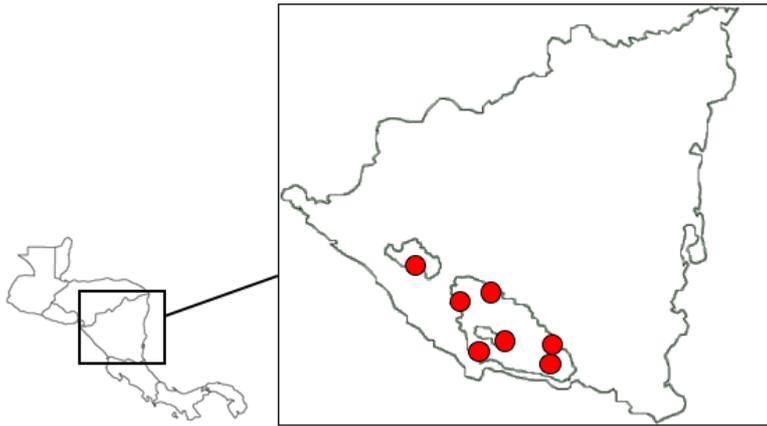
**Fig 3.** Boxplots showing the distribution of observed heterozygosity (upper panel) and nucleotide diversity (lower panel) estimated from 8038 SNPs occurring in all six populations. Note the considerable reduction of both diversity measures in the introduced Hazelwood population (Ha) compared to native Nicaraguan populations (Lv, Ma, Om, Pd and So).

## Supplementary Material

**Supplementary Table 1.** The variance in weight gain/loss in each experimental tank used in the feeding efficiency experiment. We found similar levels of variation depending on diet, with a low level of variation in shrimp diet treatments and a high level of variation in pellet diet treatments.

<b>Experimental Tank</b>	<b>Variance</b>
Tank 1 (pellet diet: rock substrate)	26.9
Tank 2 (pellet diet: sand substrate)	33.58
Tank 3 (shrimp diet: sand substrate)	0.68
Tank 4 (shrimp diet: rock substrate)	2.8
Tank 5 (pellet diet: rock substrate)	20.68
Tank 6 (shrimp diet: sand substrate)	0.45
Tank 7 (shrimp diet: rock substrate)	0.57
Tank 8 (pellet diet: sand substrate)	13.34

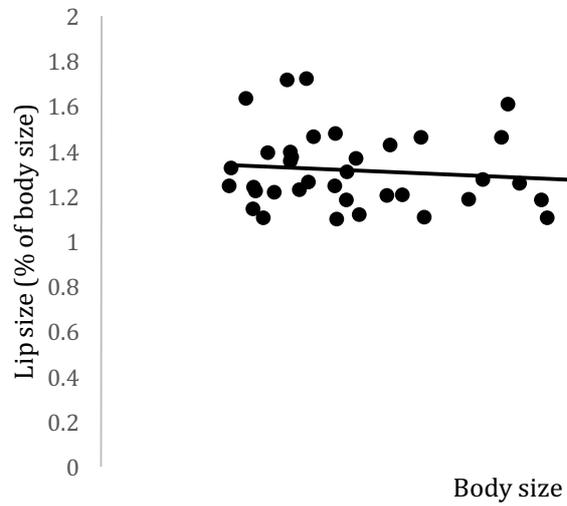
**Supplimentary Figure 1.** Sampling locations (indicated by red dots) in Lake Managua and Lake Nicaragua, Nicaragua.



**Supplimentary Figure 2.** We assessed the standardized head length of each individual by comparing the distance between the front tip of the lips and the top of the pectoral fin, with standard body length. Head width was measured downwards from the anterior starting point of the dorsal fin.



**Supplementary Figure 3.** The relationship between lip size (as a percentage of body size) and body size (area cm<sup>2</sup>) in the Hazelwood Pondage population.



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## Declaration for Thesis Chapter

Monash University

## Declaration for Thesis Chapter Three

### Declaration by candidate

In the case of Chapter Three, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conceived and designed study, Conducted fish collection and laboratory work, Analysed data, Wrote manuscript	95%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Bob Wong	Helped design study, proof read manuscript	
Topi Lehtonen	Proof read manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work\*.

Candidate's Signature		Date
		25/11/2015

Main Supervisor's Signature		Date
		25/11/2015

# Chapter Three: Background matching ability and the maintenance of a colour polymorphism in the red devil cichlid

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Published Manuscript: *Journal of Evolutionary Biology* (2015), 28 (2): 395-402



*Red devil gold and dark colour morphs, collected from Hazelwood Pondage, Victoria, Australia*

## Abstract

The evolution and maintenance of colour polymorphisms remains a topic of considerable research interest. One key mechanism thought to contribute to the coexistence of different colour morphs is a bias in how conspicuous they are to visual predators. While individuals of many species camouflage themselves against their background to avoid predation, differently coloured individuals within a species may vary in their capacity to do so. However, to date, very few studies have explicitly investigated the ability of different colour morphs to plastically adjust their colouration to match their background. The red devil (*Amphilophus labiatus*) is a Neotropical cichlid fish with a stable colour polymorphism, with the gold morph being genetically dominant and having a myriad of documented advantages over the dark morph. However gold individuals are much rarer, which may be related to their heightened conspicuousness to would-be predators. Here, we tested the ability of differently coloured individuals to phenotypically adjust the shade of their body colour and patterns to match their background. In particular, we filmed dark, gold and mottled (a transitioning phase from dark to gold) individuals under an identical set-up on light versus dark coloured substrates. We found that, in contrast to individuals of the dark morph, gold and mottled individuals were less capable of matching their body colouration to their background. As a result, gold individuals appeared to be more conspicuous. These results suggest that a difference in background matching ability could play an important role in the maintenance of colour polymorphisms.

**Keywords:**

Colour polymorphism, camouflage, Midas cichlid, predation, evolution, background matching

## Introduction

Colour polymorphisms are a striking example of biological variation and are observed across a wide range of taxa. The adaptive significance of colour polymorphisms, where two or more genetically determined colour morphs exist in a population (Huxley, 1955), remains a topic of contention. Indeed, the adaptive significance of colour polymorphs and the mechanisms facilitating their maintenance still continue to attract considerable research interest (Coyne & Orr, 2004, Gray & McKinnon, 2007, Roulin & Bize, 2007, Hancox et al., 2013). This attention has focussed mostly on the ecological significance of colour polymorphisms, as well as their potential role in sympatric speciation (Sinervo & Svensson, 2002, Gray & McKinnon, 2007, Forsman et al., 2008, McKinnon & Pierotti, 2010). Yet, in most species the exact mechanisms maintaining colour polymorphs remain unknown.

Body colour often correlates with behavioural and physiological traits, such as reproductive strategy, aggression, immune function and stress response (Barlow, 1983, Sinervo & Svensson, 2002, Pryke & Griffith, 2006, Pryke et al., 2007, Dijkstra et al., 2008, McKinnon & Pierotti, 2010). Therefore, the processes maintaining morph frequencies are likely to involve complex interactions and correlational selection. One potential difference occurring between colour morphs is their relative conspicuousness to predators. Predation pressure is a strong selective force and previous studies have demonstrated how it can contribute to the maintenance of morph frequencies (Losey et al., 1997). For

example in pea aphids (*Acyrtosiphon pisum*), predation by ladybird beetles (*Coccinella septempunctata*) interacts with parasitism from wasps (*Aphidius ervi*) to balance a red-green colour polymorphism (Losey et al., 1997). In other taxa, such as the guppy (*Poecilia reticulata*), predation impacts on the variation and distribution of colour morphs, with individuals being less conspicuous in areas where predation pressure is higher (Endler, 1980, Young et al., 2011).

In response to predators, many species have the capacity to plastically alter their body colour to match their background (Endler, 1978, Stuart-Fox & Moussalli, 2009). Habitat-specific background matching has been observed in a range of taxa, including invertebrates, fish, reptiles and birds (Reed & Janzen, 1999, Stuart-Fox et al., 2004, Whiteley et al., 2009). For example, in the freshwater sculpin (*Cottus aleuticus*), body colour is significantly correlated to local substrate colour, and sculpins can alter their body colour to match new backgrounds in a matter of months (Whiteley et al., 2009). In contrast, rapid background matching, a process most likely under neural control, is well documented in cephalopods and has also been observed in fish and reptiles (Hanlon & Messenger, 1988, Stuart-Smith et al., 2008, Hanlon et al., 2009, Clarke & Schluter, 2011). Background matching ability has important ecological and evolutionary implications, with slow or less precise individuals potentially more likely to be noticed by predators (Endler, 1980). In the context of colour morphs, differences in the ability to background match – and hence avoid detection by would-be predators – have the potential to play an important role in maintaining polymorph frequencies. Nevertheless, morph-specific responses to predation, for example via camouflage, have been largely unexplored.

The Midas cichlid species complex (*Amphilophus* spp.) is a group of closely related Neotropical fish, several of which are polymorphic in regard to body colour (Elmer et al., 2010c). In these polymorphic species, the majority of individuals are “dark”, while approximately 10% of individuals are “gold” in body colour (yellow through red; Meek, 1907, Barlow, 1973, Barlow & Munsey, 1976). All individuals begin life with the dark body colouration. However, genetically ‘gold’ individuals generally start to change colour, from around three to 12 months of age, i.e., with many ‘gold’ fish starting the transition prior to reaching sexual maturity. This occurs when fish begin to lose the melanophores in their skin, which then exposes the underlying gold colouration – a process that can take several days to months to complete (Barlow, 1983, Dickman et al., 1988). Gold colouration has a dominant genetic inheritance and some intraspecific advantages of being gold have been demonstrated, such as, an increased growth rate of gold-coloured individuals when raised with dark individuals, less aggression being directed to gold-coloured individuals when they comprise less than 50% of the population, as well as gold individuals dominating dark ones of equal size in dyadic encounters (Barlow, 1973, 1983, Lehtonen, 2014). However, despite the advantages, there is still no clear explanation as to why gold individuals are so clearly less common than dark individuals in native *Amphilophus* populations (Barlow, 1976). Interestingly, in an introduced population in Australia with relaxed natural predation pressure, gold individuals are more common than their dark conspecifics, making up approximately 65-70% of the population (Sowersby, Lehtonen and Wong; unpublished data).

It has earlier been noted that individuals of the dark morph have an ability to alter the shade of their body colour and their patterning, a process that is limited in genetically gold individuals, even as juveniles (Barlow, 1976, Dickman et al., 1990). Nevertheless, while a handful of studies have considered a difference in predation level as a factor in maintaining the Midas cichlid colour polymorphism (Annett, 1989, Dickman et al., 1990, Maan et al., 2008, Maan & Sefc, 2013, Kusche & Meyer, 2014, Torres-Dowdall et al., 2014 ), potential differences between the colour morphs in their ability to alter their colouration to better match their background have so far been largely overlooked. In this study, we used the colour polymorphic red devil (*Amphilophus labiatus*, Günther, 1864) to test the background matching ability of individuals of the two colour morphs against contrasting natural backgrounds. We hypothesised that dark individuals would be better able to alter their body colour/shade to match their surrounding background than gold individuals. Such a difference in ability to adjust their colour/shade would allow individuals of the dark morph to more closely match their background, leaving individuals of the gold morph more conspicuous and potentially more susceptible to would-be predators – thus helping to explain their low frequency in nature.

## Methods

### *Study species*

*Amphilophus labiatus* is a Neotropical fish native to the great lakes of Nicaragua (Lake Nicaragua and Lake Managua). The species has been introduced into Australia within the last 40 years, where it has established a breeding population in the man-made Hazelwood Pondage in south eastern Australia (NIWA, 2008). The fish used in this experiment ( $N=70$ ) were collected from Hazelwood and transported to Monash University, where they were maintained in four large, bare bottomed flume tanks ( $\sim 3000$  l) at a temperature of  $24^{\circ}\text{C}$  on a 12:12 day/night cycle and fed daily on commercial fish food pellets. All fish used were adults or sub-adults, between 12 and 16cm total length.

### *Capturing background matching on natural substrates*

To address the question of whether colour morphs differ in their ability to match their background, we set up glass tanks (150L), with either dark or light substrate. Dark substrate consisted of fine gravel, pebbles and small cobbles while light substrate contained sand and pebbles. Within each tank, a smaller area was created using clear Perspex sheeting ( $20\text{cm} \times 10\text{cm} \times 30\text{cm}$ ) in order to constrain fish close to the front of the tank. Fish were placed individually into the smaller area and allowed five minutes to acclimate. Using a digital camera (**Nikon D5200 Digital SLR**), fish were video recorded in high definition for a period of 30 seconds, with manual white balance and identical camera settings for each fish. Digital images are commonly used to quantify colouration in a

range of taxa (Stevens *et al.*, 2007) including fishes (Whiteley *et al.*, 2009; Morrongiello *et al.*, 2010; Clarke & Schluter, 2011, Hancox *et al.* 2013, Stevens *et al.* 2014). A camera's response to light can be highly dynamic. Therefore, before each session, it was important to test the camera's light meter and white balance. We chose to film (rather than photograph) the fish because filming allowed us to 1) more easily capture a free swimming fish in focus, and 2) had a higher signal to noise ratio, compared to a single shot (unpublished data). A colour-checker card (ColorChecker, X-rite Inc.) and white and grey standards (Pro Photography Digital) were included in a sub-set of the photographs (see Whiteley *et al.*, 2009; Morrongiello *et al.*, 2010; Clarke & Schluter, 2011). We were then able to compare these standards (grey reflectance and white standard) within and between images. We found that the response of red, green and blue (RGB) colour channels was not appreciably different with respect to the grey standards and that any variation we did see was extremely minor (less than 2% difference between images) compared to the variation that existed, both within and between fish.

Lighting was provided by three white LED (light-emitting diode) lights, one at a perpendicular angle to the test tank and the other two elevated and at a 45-degree angle. LED lights have been used successfully in the past (Svensson 2007), as they provide a stable colour temperature and light intensity over time. A total of 23 dark individuals (i.e. fish with no gold pigmentation on the body), 20 mottled individuals (i.e. fish in the process of transitioning to gold colour, with approx. 50% gold and 50% dark pigmentation; Dickman *et al.*, 1988, which constitute approximately 10% of the Australian population) and 27 gold

individuals (i.e. fish that had completely transitioned, with no dark spots on the body) were filmed on both light and dark substrates. To do so, each fish was transferred between substrate treatments, where we repeated the acclimation period and filming procedure. We randomly assigned half of the fish to be filmed on dark substrate first followed by the light substrate; with the remainder filmed in the reverse order. The experimental tanks were filmed without fish, haphazardly before, after and between replicates ( $n=20$ , per substrate type). This was done so that the RGB values of the two substrate treatments could be quantified (see below) and as an additional confirmation, along with colour-checker and standard cards, that lighting conditions did not alter over time.

### ***Analysis of digital images***

A single frame from each video (RAW format) was selected where the left lateral side of the fish was in focus and imported into ImageJ 1.47v (Kelley et al. 2012). The sum of the RGB scores were assessed (similar to Clarke & Schluter, 2011) at six (80 pixels each) identical points on each fish, for each image: four points from the dorsal side, the top lip, upper eye, start of the dorsal fin and upper tail and two from the ventral, lower eye and at the base of the pectoral fin (Fig. 1). In order to assess typical brightness of the two substratum/background types, RGB scores were assessed at five (80 pixels each) identical points in 20 images.

### ***Statistical Analysis***

The sum of the RGB scores were assessed at the six points on each image (Fig. 1), with each fish being recorded twice, once on dark and once on light substrate. First, to determine if the RGB scores (averaged over the 80-pixel point size) differed between the three colour types and two substrate types, we analysed each of the six measurement points separately using a split-plot ANOVA with fish colour and substrate type as categorical variables. Because each individual was measured on both substrate types, the identity of each individual fish was treated as a random factor. We then did a test of the main effects at each point to determine if color morphs differed between substrates in regard to RGB scores. Secondly, to assess how well the differently coloured individuals matched the two substrate types, we applied a series of one-way ANOVAs, each comparing the RGB scores of the following five groups: the three fish colour types and of the two substrate types. Each of the six measurement points was again analysed separately. To test which pairs of groups were significantly different from each other, we then applied post-hoc unplanned pairwise Tukey's comparisons. A lack of a significant difference between a fish colour type and substratum type would imply an accurate background matching, whereas a significant difference would suggest a mismatch. Finally, to test for differences in brightness of the two substrate types, their RGB scores were compared using a two-sample t-test. We used R 3.0.0 software (R Development Core Team) for all analyses.

Collection and experimental procedures were approved by the Animal Ethics Committee of Monash University, Australia (BSCI/2012/23) and complied with all relevant State and Federal laws.

## Results

Firstly, we found that for each of the three types of individuals, there was a significant interaction between substrate type and the brightness of body colour at each point on the dorsal side of the body (Table 1), indicating that body shade/colouration changed on different coloured substrates. By contrast, there was no significant interaction on the ventral side of the body, at the lower eye, and pectoral fin points (Table 1). We found that dark individuals became significantly lighter on light substrate compared to dark substrate at all points taken from the dorsal side of the fish (Fig. 2). Conversely gold and mottled individuals showed the opposite relationship, displaying darker colouration on light substrates than on dark substrates, except for the dorsal fin point of mottled morphs where there was no significant difference (Table 2).

On the ventral side of the body, dark individuals at the pectoral fin point did not differ significantly from light and dark substrates, gold individuals did significantly become darker on light substrate and lighter on dark substrate, mottled individuals did not differ significantly from light and dark substrates (Fig. 2). At the lower eye point on the ventral side dark individuals did differ significantly from light and dark substrates, gold individuals became darker on light substrate and lighter on dark substrate, mottled individuals did not differ from light and dark substrates (Table 2).

Secondly, pairwise comparisons revealed that on dark substrate, dark individuals did not significantly differ from their background on the dorsal side,

except for the dorsal fin, but did differ from the background on the ventral side of the body (Table 3). By contrast, gold and mottled individuals significantly differed from both the dark and light substrates on all of the measured parts of the body (Table 3).

Finally, we found that the dark substrate was significantly darker than the light substrate (Mean  $\pm$  s.e.; RGB score of dark substrate =  $48.1 \pm 3.6$ , light substrate =  $143.3 \pm 3.5$ ; Two-sample t - test,  $t_{86} = 18.91$ ,  $p = 0.0001$ ).

## Discussion

In this study we investigated the background matching ability of the colour polymorphic cichlid, *A. labiatus*. We found a difference in the background matching ability of the different colour morphs. Dark individuals, which are more abundant in natural populations, significantly altered the shade of their body colour between substrates, being lighter on light coloured substrate and darker on dark substrate. Gold individuals, less common in natural populations, along with transitioning (mottled) individuals, did not alter their body to match their background substrate. Instead, gold and mottled individuals tended to become darker on light substrate and lighter on dark substrate.

*Amphilophus* cichlids are common and widespread in Nicaragua and individuals

regularly traverse between dark volcanic rocks to lighter sand and silt habitats, in both the two large “great” lakes (Lake Nicaragua and Lake Managua) and the smaller and more numerous crater lakes (Barlow & Munsey, 1976, Cole, 1976). A strategy for avoiding predators across habitats could involve colour matching against a range of backgrounds, therefore an individual ought to express a phenotype that is optimal for camouflage in varied environments by altering its body colour (Wente & Phillips, 2003, Ruxton et al., 2004). We found that dark coloured individuals altered the brightness of their body colour to correspond to the substrate they were on, which was first suggested (but not formally investigated) by Barlow (1976). We found that, quantitatively, background matching of the dark morph was not perfect. Instead dark individuals may be employing a “compromise” strategy, matching the specific brightness of common backgrounds and more generally of other less frequently encountered backgrounds, rather than being specialised and matching one type of background. The dark morph did not alter the dorsal and ventral sides of the body equally, with the ventral side of the body generally always being lighter and having a less dramatic change across substrate types. This pattern is consistent with counter shading, a noted camouflage strategy, which individuals employ to reduce the chance of detection by predators or prey (Thayer, 1896, Stevens & Merilaita, 2009). To be effective, a camouflage or background-matching pattern needs to be a representation or approximation of a sample of the backgrounds normally viewed by predators (Endler, 1978, Merilaita et al., 1999, Houston et al., 2007). This may be an efficient strategy for dark individuals, allowing them to camouflage against a range of backgrounds as they move through a heterogeneous environment (Barlow, 1976; Stevens & Merilaita, 2009).

As hypothesised, we did not observe gold individuals (or transitioning mottled individuals) altering the shade of their body colour in a manner that would have matched background substrates. Instead, gold individuals appeared to darken their body on light substrate (background) compared to on dark substrate. This result was unexpected, especially due to the general conspicuousness of gold individuals. Regardless, it appears that gold individuals do not have the same cryptic ability as dark individuals and are, in general, more conspicuous. Being more visually conspicuous has been shown to increase predation risk and, importantly, has been shown to influence colour morph distributions in other species of fish (Endler, 1978, Endler, 1980, Goodwin et al., 1998, Young et al., 2011).

Disparity in predation rate between colour morphs is considered to be important in contributing to the maintenance of stable polymorph frequencies (Losey *et al.*, 1997). The results from our study show that in *A. labiatus*, individuals of the dark morph are able to plastically adjust the shade of their body colour in response to different substrate colour much more effectively than the gold morph. Indeed, evidence suggests that differences in background matching ability may be present in dark and gold individuals even before the transition process has begun, with Dickman *et al.* (1990) reporting that genetically gold *Amphilophus* juveniles have poorer control over their body colour and markings than genetically dark individuals.

The lakes of Nicaragua contain several large predators capable of consuming

small to mid sized *Amphilophus* cichlids, including piscivorous fish, birds and caimans (Davies, 1976, Blake, 1977). Difference in predation pressure in *Amphilophus* cichlid morphs has been suggested previously (Annett, 1986, 1989, Dickman et al., 1990) and do not appear to be due to any behavioural differences in antipredator response. More recently, conspicuousness of gold individuals to both fish and bird predators has also been demonstrated experimentally (Kusche & Meyer, 2014; Torres-Dowdall *et al.*, 2014). It is important to bear in mind, however, that the susceptibility of dark and gold individuals to different predators is likely to depend on a range of factors, such as the predator's visual acuity, their mode of attack, and the environmental conditions in which the animals occur (e.g. turbidity) (Stuart-Fox et al. 2006). Hence, more work is needed to understand how differences in colouration might influence susceptibility of dark and gold individuals to different predators and under different environmental conditions.

More generally, the maintenance of a stable colour polymorphism is likely to be the result of complex interactions, including behavioural and physiological differences, with our results suggesting that one such difference is a background matching disadvantage of individuals of the gold morph. These behavioural and physiological traits may play a role in maintaining colour polymorph frequencies, via correlated selection, compensating for colour morph disadvantages in certain circumstances (Sinervo et al., 2001, Lank, 2002, Hadfield et al., 2007, Gray & McKinnon, 2007). For example in Gouldian finches (*Erythrura gouldiae*), different colour morphs employ distinct behavioural strategies, particularly in regard to aggression, which in turn leads to differences

in hormone expression and immune performance (Pryke et al., 2007). In addition, the aggressive red morph finches suffer more greatly from stress in social situations where they are not rare and females have elevated stress when they are paired with incompatible mates, i.e. non-like colour morphs (Pryke et al., 2007, Pryke et al., 2011). It is also likely that multiple mechanisms are acting to maintain the *Amphilophus* cichlid colour polymorphism. However, in the introduced Australian population, gold morphs exist at a much higher frequency than in their native range, over 60%, compared to less than 20% (Elmer et al. 2010). This may be due to a founder effect and/or individuals being potentially freed from the assemblage of key predators that occur in their native habitat, although more work is needed to test these possibilities.

In summary, *A. labiatus* colour morphs differed in their background matching ability in response to different substrates. Together our results suggest that differences in the ability to background match could play a potentially important role in maintaining colour polymorphism frequencies in the wild.

## Tables and Figures

**Table 1** Output of split-plot ANOVAs, investigating interactions between substrate type and fish colour morph

Point	Df	F-value	P-value
Lip	2	12.26	<0.0001
Upper Eye	2	14.7	<0.0001
Dorsal Fin	2	19.06	<0.0001
Tail	2	22.5	<0.0001
Lower Eye	2	2.69	0.07
Pectoral Fin	2	0.84	0.43

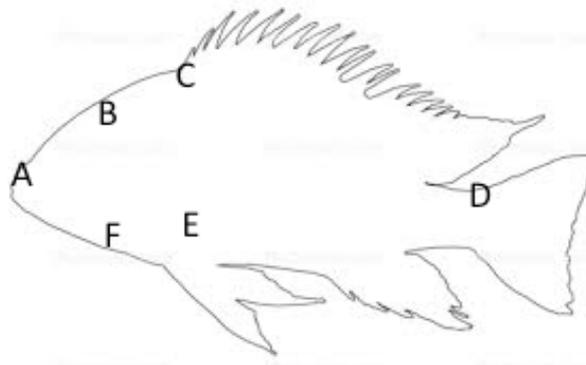
**Table 2** Output of main effects test at each point to determine if color morphs differed between substrates in regard to RGB scores

Point	Gold Morph			Dark Morph			Mottled Morph		
	Df	t-value	P-value	Df	t-value	P-value	Df	t-value	P-value
Lip	134	-2.58	0.01	134	4.3	<0.001	134	2	0.04
Upper Eye	134	-2.78	0.006	134	4.93	<0.0001	134	1.801	0.007
Dorsal Fin	134	4.15	<0.0001	134	4.15	<0.0001	134	1.19	0.2
Tail	134	-3.42	<0.001	134	6.09	<0.0001	134	2.45	0.01
Lower Eye	134	-2.78	0.006	134	4.93	<0.0001	134	1.8	0.07
Pectoral Fin	134	0.29	0.76	134	2.04	0.04	134	1.75	0.08

**Table 3** Output of one-way ANOVA with an unplanned pairwise comparison comparing RGB scores from colour morphs to dark substrate RGB scores and to light substrate RGB scores

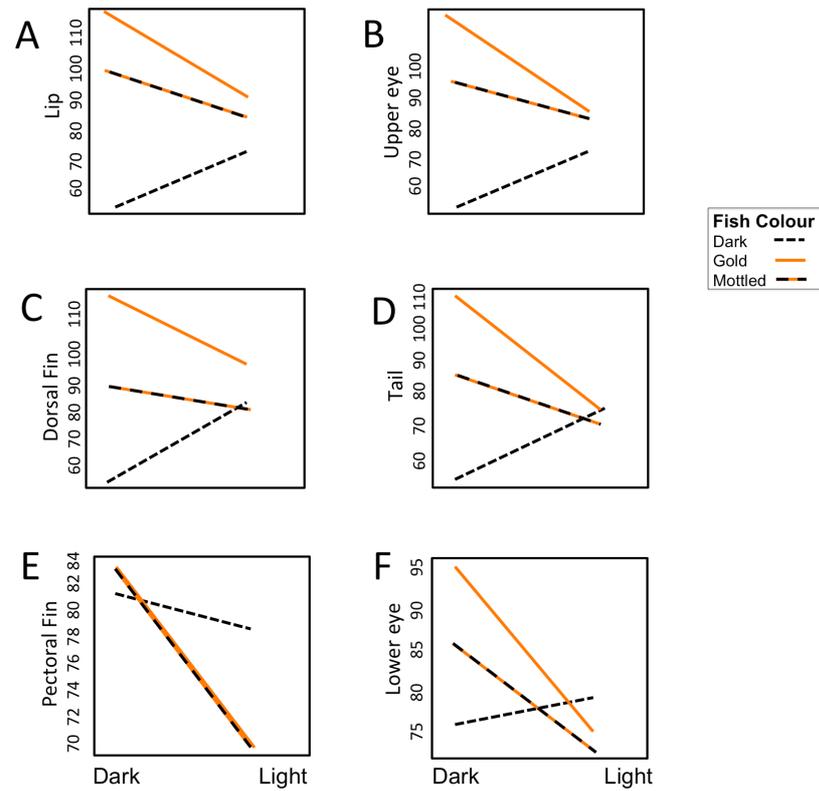
Point	DF	Gold Morph				Dark Morph				Mottled Morph			
		Light Substrate		Dark Substrate		Light Substrate		Dark Substrate		Light Substrate		Dark Substrate	
		t-value	P-value	t-value	P-value	t-value	P-value	t-value	P-value	t-value	P-value	t-value	P-value
Lip	219	10.39	<0.00001	-13.67	<0.00001	-13.67	<0.00001	12.74	0.156	6.38	<0.00001	-7.04	<0.00001
Upper Eye	219	10.21	<0.00001	-3.93	<0.00001	-12.68	<0.00001	0.72	0.164	6.83	<0.00001	-6.42	<0.00001
Dorsal Fin	219	11.44	<0.00001	-2.64	<0.00001	-12.48	<0.0001	0.84	0.0032	5.709	<0.00001	-7.03	<0.00001
Tail	219	10.57	<0.00001	-5.9	<0.00001	-14.82	<0.0001	0.88	0.06	5.93	<0.00001	-9.02	<0.00001
Lower Eye	219	6.89	<0.00001	-6.97	<0.00001	-9.12	<0.00001	3.9	<0.0001	4.907	<0.00001	-7.56	<0.00001
Pectoral Fin	219	7.02	<0.00001	-8.44	<0.0001	-8.37	<0.00001	4.42	<0.00001	5.709	<0.00001	-7.65	<0.0001

**Figure 1**



**Fig. 1** Points on each fish where RGB scores were taken on both light and dark substrates: (A) top lip, (B) above the eye, (C) at the start of the dorsal fin, (D) upper tail (on the peduncle before the caudal fin), (E), pectoral fin (at the base of the fin) and (F) below the eye

Figure 2



**Fig. 2** RGB scores taken at six identical points on each fish on two different coloured substrates, dark and light. (A) top lip, (B) upper eye, (C) start of the dorsal fin, (D) tail fin, (E) top of the pectoral fin, and (F) below the eye. Lower RGB scores indicate darker colour.

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## SECTION TWO

## Declaration for Thesis Chapter

Monash University

## Declaration for Thesis Chapter Four

### Declaration by candidate

In the case of Chapter Four, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conceived and designed study, Conducted fish collection and behavioural experiments, Analysed data, Wrote manuscript	95%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Bob Wong	Proof read manuscript	
Topi Lehtonen	Aided with statistical analysis, proof read manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work\*.

Candidate's Signature		Date
		25/11/2015

Main Supervisor's Signature		Date
		25/11/2015

# Chapter Four: Temporal and sex-specific patterns of breeding territory defence in a colour polymorphic cichlid fish

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**Submitted Manuscript**



*A red devil breeding pair*

## Abstract

In biparental species, the costs and benefits of parental investment can vary between the sexes or shift over time. However, such temporal and sex-specific changes in territory defence are not well understood. Here, we experimentally investigated parental investment in breeding territory defence in a feral population of colour polymorphic, biparental cichlid fish, the red devil (*Amphilophus labiatus*). We presented either gold or dark coloured conspecific intruder models (i.e. dummy models) to *A. labiatus* pairs at three key stages during the breeding cycle (i.e. after pair formation, after eggs have been laid, and when fry were free-swimming). We found that males were more aggressive when the pair first formed, whereas females significantly increased their territory defence with time, and were most aggressive when fry were free swimming. These results show that parental roles in territory defence can markedly shift over key stages of the breeding cycle. Together, our results demonstrate that parental behaviours may not only vary between the sexes, but can also shift dramatically over the course of the brood cycle.

### **Keywords:**

Aggression, colour polymorphism, parental care, sexual conflict, territoriality, cichlid fish, introduced species

## Introduction

A key component of parental care in many species is the aggressive defence of a breeding territory and offspring against intruders, such as conspecific and heterospecific competitors and predators (Ridley, 1978; Blumer, 1979; Perrone & Zaret, 1979). For example, more aggressive red-backed shrikes (*Lanius collurio*) have better reproductive success than less aggressive individuals (Tryjanowski & Golawski, 2004). Aggressively defending breeding territories can therefore play a key role in parental fitness and offspring success.

Benefits aside, guarding young and breeding territories can also be costly for the parents in terms of increased energy expenditure (Haller, 1996), reduced foraging opportunities (Requena et al., 2012), heightened risk of injury and mortality (Marler & Moore, 1988, Lappin & Husak, 2005), as well as lost future mating opportunities (Trivers, 1972, Székely & Cuthill, 2000). For instance, when female crickets (*Oecanthus nigricornis*) are carrying eggs, they are significantly more likely to be taken by predatory wasps (*Isodontia mexicana*; Ercit et al., 2014). Yet, to date, most studies of parental behaviours have tended to focus on offspring provisioning (predominately in birds Harrison et al., 2009), with relatively less attention given to the role of territorial defence and offspring guarding in shaping the relative investment of the sexes in offspring care (Clutton-Brock 1991; Székely & Cuthill, 1999; Harrison et al., 2009; Trnka & Grim, 2012).

When both parents contribute to care, the costs and benefits are often distributed unevenly between the sexes. Biparental care therefore involves elements of not only cooperation, but also conflict (Lessells, 1999; Chapman et al., 2003; Houston et al., 2005; Harrison et al., 2009). In many species, potential conflicts between the sexes can arise, for example, due to differences between the potential reproductive rates of males and females (Baylis, 1981; Reynolds, 1996), or because males may lack assurance over the paternity of the offspring they are raising (Trivers, 1972; Keenleyside, 1991). As a result, the level of care provided by parents is often not shared equally (Trivers, 1972; Wynne-Edwards, 1995), and may change over time, with a parent sometimes even deserting its partner before offspring become independent (Keenleyside, 1983; McNamara et al., 2002). Despite this, surprisingly few studies have taken an experimental approach to investigate how the sexes might alter their investment in parental behaviours, such as territory defence, over the course of the breeding cycle.

One large group of vertebrates, the cichlid fishes (Cichlidae), show remarkable interspecific variation in their forms of parental care. For this reason, cichlid fishes are well suited for testing hypotheses on the evolution of vertebrate parental care (Goodwin et al. 1998). Offspring guarding and territory defence, against both heterospecific and conspecific egg and fry predators, is the most common form of parental care in fishes, including cichlids (Gross and Sargent 1985). Accordingly, we experimentally investigated parental roles in well-defined phases of the brood cycle in a colour polymorphic Neotropical cichlid fish, the red devil (*Amphilophus labiatus*, Günther, 1864). *Amphilophus labiatus* is endemic to the two great lakes of Nicaragua, Lake Managua and Lake Nicaragua,

however it has been introduced elsewhere due to its popularity in the aquarium trade. Like other members of the Midas cichlid complex (Barlow, 1976; Rogers, 1988; McKaye & Murry, 2008; Elmer et al., 2009; Lehtonen et al., 2012), *A. labiatus* displays biparental care and is sexually monomorphic with respect to colour. When pairs are ready to spawn, they claim a breeding territory, which they actively defend against intruders (McKaye, 1977; Rogers, 1987; Barlow, 2000). In the wild, fry of *Amphilophus* cichlids are highly vulnerable to predators and require the parents' protection for survival from both heterospecifics and conspecifics (Barlow, 1976; Rogers, 1987; McKaye & Murry, 2008; Lehtonen et al., 2012). During this period, pairs must also subsist almost entirely upon accumulated fat reserves, which further adds to the costs of parental care. The ability to successfully defend young is therefore critical to reproductive success (McKaye, 1977; Rogers, 1987, 1988; Barlow, 2000). Although a number of studies have previously investigated sex differences in parental care investment and territory defence in Neotropical cichlids (Keenleyside et al., 1990; Budev et al., 1999; Itkowitz et al., 2001; Wisenden et al., 2008), including in close relatives of *A. labiatus* (Holder et al. 1985; Rogers, 1988; MacKaye & Murry, 2008), it is surprising that few have experimentally controlled either the appearance of territorial intruders or the exact timing of territorial intrusions throughout a breeding period. Such experimental manipulations, however, are important if we are to gain a more comprehensive understanding of why the sexes might differ in their investment in territory defence, especially as this is a key aspect of parental care in these fish.

The specific aim of the current study was to investigate how males and females alter their investment in territory defence and offspring guarding at three key stages of the breeding cycle.

## **Methods**

### ***Collection and housing***

*Amphilophus labiatus* were collected using hand-lines in late 2013 from a feral population in Hazelwood Pondage, in south-eastern Australia, where they have been introduced and have established a breeding population over the last 40 years (NIWA 2008). Fish were transported to Monash University and housed in large stock tanks (4 x 4000 litres, 26°C, 12:12 day night cycle, stocking density of one fish per 33 litres) furnished with gravel, rocks and PVC pipes for shelter. All fish were fed commercial cichlid pellets (Otohime EP3) daily.

### ***Pair formation***

To assess breeding territory defence, we first needed to allow individuals to naturally form into breeding pairs. This was done by randomly selecting 6 similarly sized mature fish and putting them together into large experimental tanks of ~1000L, supplied with terracotta pots as potential spawning sites. A pair bond was deemed to have formed when two individuals were observed interacting around a potential spawning site. At this point, all other fish were removed from the experimental tank. During the course of the study, we were

successful in obtaining eleven pairs, eight of which paired assortatively based on body colour (i.e. four gold-gold pairs and four dark-dark pairs) and three non-assortatively, which gave us a total of 11 gold and 11 dark individuals. Each pair was used only once. Fish were measured (standard length mm) at the end of the experiment.

### ***Intruder models***

We simulated aggressive encounters in a controlled fashion by using models of territory intruders presented to our focal pairs. Each model mimicked either a dark or gold *A. labiatus* (conspecific) territory intruder. We used models of conspecific territory intruders because *A. labiatus* is the most abundant species in Hazelwood Pondage and is therefore likely to present a high predation risk to both eggs and juveniles. Additionally, we have found both fish eggs and fish remains in the gut content of *A. labiatus* individuals from both Hazelwood Pondage and from native Nicaragua populations (unpublished data). Moreover, previous studies have also used conspecifics as intruders to successfully elicit aggression in the context of breeding territory defence in other cichlid species, closely related to *A. labiatus* (Holder et al. 1991; Itzkowitch 1985).

In common with many other members of the Midas species complex, *A. labiatus* has a genetically distinct colour polymorphism, with both dark (i.e. grey through to black) and gold (yellow through red) coloured individuals (Barlow, 1983; Elmer et al., 2010). In such systems, biased aggression towards particular individuals, or inherent differences in aggressiveness between phenotypes (e.g. colour morphs) could play an important role in the evolution and maintenance of

colour polymorphisms, as observed in Gouldian finches (*Erythrura gouldiae*) (Pryke, 2007) and side-blotched lizards, (*Uta stansburii*) (Sinervo et al., 2000). We therefore created models of both colour morph to test whether the level of aggressive territory defence provided by a pair is influenced by the colour of the territory holders and/or conspecific territorial intruders.

“Dummy” models have been successfully used as stimuli to experimentally elicit behaviours in a wide range of fish species (Rowland, 1999), including *Amphilophus* cichlids – both in the field and in the laboratory (Barlow & Siri, 1994; Lehtonen, 2014; Lehtonen et al., 2015a, 2015b). We decided to use models instead of live stimulus animals to enable us to control for possible confounding factors that might arise from differences in the behaviour of stimulus animals. As with recent studies on other *Amphilophus* species (Lehtonen, 2014; Lehtonen et al., 2015a, 2015b), we created realistic looking models based on photographs of actual fish, rather than the more stylized models that have traditionally been used in the majority of earlier studies (e.g. Barlow & Siri, 1994; Rowland, 1999).

Specifically, each of the models was made using waterproof, photographic colour prints of the lateral side of a live *A. labiatus* individual. These images (length= 180mm) were then glued onto both sides of a fish-shaped plastic PVC foam plate (thickness = 6 mm; Supplementary Fig. 1; also see Lehtonen 2014). Each model was attached to a sinker with a fishing line, which allowed it to float in a natural position approximately 15 cm above the tank bottom (as per Lehtonen, 2014).

### ***Experimental protocol***

We used in total eight gold and eight dark intruder models to simulate conspecific territory intruders, with each model based on a photograph of a different *A. labiatus* individual. Individuals whose image was used to create a stimulus model were also excluded from use as potential focal fish (and vice versa). One model was introduced to the focal fish two hours after the pair first formed (additional fish were previously removed from the tank), another one the day after eggs had been laid, and the third model was introduced on the day after fry were observed free swimming, with a different stimulus model used on each occasion. Thus, each focal pair was exposed to a unique combination of three models (with the colour of the model in a given presentation being randomised). The three distinct phases for model presentation were chosen to represent key stages in the breeding cycle to allow us to test for differences (if any) in parental investment by males and females over time.

Each replicate was initiated by placing a model approximately 40 cm from the centre of the *A. labiatus* breeding pair's territory. After an acclimation period of 30 seconds, we counted the total number of mobile aggressive behaviours (charges and bites) directed by each territory owner (male and female) towards the model over a two-minute observation period, which allowed us to calculate a total 'aggression rate' for each pair and each individual (*sensu* Lehtonen, 2014; Lehtonen et al., 2015a, 2015b). All trials were filmed with a camcorder positioned on a tripod and watched live, on a closed circuit system. The trials were run between January and March 2014.

### ***Statistical analysis***

To assess the patterns of parental aggressive behaviour, we used a generalized mixed model with a negative binomial error distribution. In particular, the full model was fitted with reproductive stage (i.e. pair bond, eggs, fry) and sex of the territory owner as explanatory fixed factors, and with size of the territory owner, colour of the dummy model intruder and colour (morph) of the territory holder as covariates. To account for the paired design of the experiment (i.e. multiple stimulus presentations to the same pair and to the same individuals) and the potential interdependence between the actions of the paired female and male defending the same territory, both “pair ID” and “individual ID” were added as random effects (as per Pinheiro & Bates 2000; Lehtonen et al. 2015a).

To assess whether there was an effect of reproductive stage on male and female patterns of parental aggressive behaviour, we then used two separate (one for each sex) generalized mixed models with a negative binomial error distribution. Both models were fitted with reproductive stage as a fixed explanatory factor and with size of the territory owner, colour of the dummy model intruder and colour (morph) of the territory holder as covariates. To account for the paired design of the experiment (i.e. multiple stimulus presentations to the same pair) and the potential interdependence between the actions of the paired female and male defending the same territory, “pair ID” was added as a random effect in both models. Both full models were simplified by stepwise removal of non-significant interaction terms (Crawley 2007), using log likelihood ratio tests (with  $\alpha = 0.05$ ). We used R 3.0.0 software (R Development Core Team) for all analyses.

## Results

In total, model intruders elicited aggressive responses in 94% of females and 88% of males. The average standard body length of females was 16.8 cm (range= 14.9-19.7 cm) and 18.6 cm (range= 15.9-21.3 cm) for males.

When we applied a generalized mixed model to assess the effects of the reproductive stage and the sex of the focal territory holders on the rate of aggression, we found a significant interaction between the stage of the reproductive cycle and the sex of the individual ( $t_{57}=-6.85$ ,  $p<0.00001$ ) on the level of aggression. That is, males and females differed in their rates of aggression depending on the stage of the reproductive cycle (Fig. 1). We found no effect of the covariate variables, size of the territory owner ( $t_{57}=1.52$ ,  $p=0.13$ ), colour of the dummy model intruder ( $t_{57}=-1.63$ ,  $p=0.1$ , Fig. 2) or colour (morph) of the territory holder ( $t_{57}=-1.02$ ,  $p=0.31$ , Fig. 2) on patterns of parental aggression.

Next, when we applied a generalized mixed model to assess the effect of reproductive stage on patterns of male parental aggressive behaviour, after model simplification, we found a significant effect of reproductive stage on male aggressive behaviour ( $z_2=48.45$ ,  $p<0.00001$ , Fig.1.). The covariate variables, size of the territory owner ( $z_1=0.59$ ,  $p=0.43$ ), colour of the dummy model intruder ( $z_1=0.042$ ,  $p=0.83$ ) and colour (morph) of the territory holder ( $z_1=-1.92$ ,  $p=0.16$ ) had no significant effect on patterns of male parental aggression.

Similarly, when we applied a generalized mixed model to assess the effect of

reproductive stage on patterns of female parental aggressive behaviour, after model simplification, we found a significant effect of reproductive stage on female aggressive behaviour ( $z_2=53.07$ ,  $p<0.00001$ , Fig.1.). The effect of the covariate variables, size of the territory owner ( $z_1=2.63$ ,  $p=0.1$ ), colour of the dummy model intruder ( $z_1=1.5$ ,  $p=0.21$ ) or colour (morph) of the territory holder ( $z_1=0.03$ ,  $p=0.85$ ) was not significant on patterns of female parental aggression.

## Discussion

We observed a significant shift in parental investment in territory defence over the course of the breeding cycle. In other words, investment in territory defence at key stages was not divided equally between the sexes. In particular, females were most aggressive towards territorial intruders later in the breeding cycle, when fry were free swimming. In contrast, the level of territorial defence displayed by males was highest when the pair had first formed and subsequently tapered with time. We also found that the size of a territory owner did not have a significant effect on the level of territory defence, with larger individuals being more aggressive towards territorial intruders. Moreover, in the current study, we found no evidence that territorial aggression was significantly affected by the colour of the intruder or parent.

A temporal increase in parental investment by females is concordant with studies in other taxa, such as birds, in which most of the research has so far been carried out (Schipper, 1973; Newton, 1979; Carere & Alleva, 1998; Watts, 2014).

For example, female common swifts (*Apus apus*) attend to nests and offspring at a higher rate than males from the nestling stage onwards (Carere & Alleva, 1998). The results of our current experimental study are also in accordance with observational findings in some other cichlid species (McKaye, 1977; Rogers, 1988; McKaye & Murray, 2008). In particular, these previous studies suggest that females of biparental cichlids focus less on territory defence and more on other parental activities in the early stages of the breeding cycle, while males specialise in territorial defence right from the onset of the brood cycle (Rogers, 1988; Murry et al., 2001; Itzkowitz et al., 2005; McKaye & Murry, 2008).

We provide two explanations to account for the subsequent increase in territorial defence intensity by females (*sensu* Redondo & Carranza, 1989). First, females may be compensating for temporal changes in susceptibility of offspring to predation. That is, Midas cichlid fry become more vulnerable to predators when they start to swim actively (the free swimming phase) and consequently require continual protection for survival (McKaye, 1977; McKaye & Murry, 2008). Second, the reproductive value of offspring increases as the breeding cycle progresses. Specifically, older offspring are more valuable to parents due to their increased probability of reaching maturity and the parental investment that would be required to replace them (Salfert & Moodie, 1985; Rytönen et al., 1995; Jaroensutasinee & Jaroensutasinee, 2003). Females may therefore be adjusting their level of territory defence as a direct response to these specific selection pressures, particularly as males are simultaneously lowering their level of territorial defence at this stage (*sensu* Hammerstein & Parker, 1987).

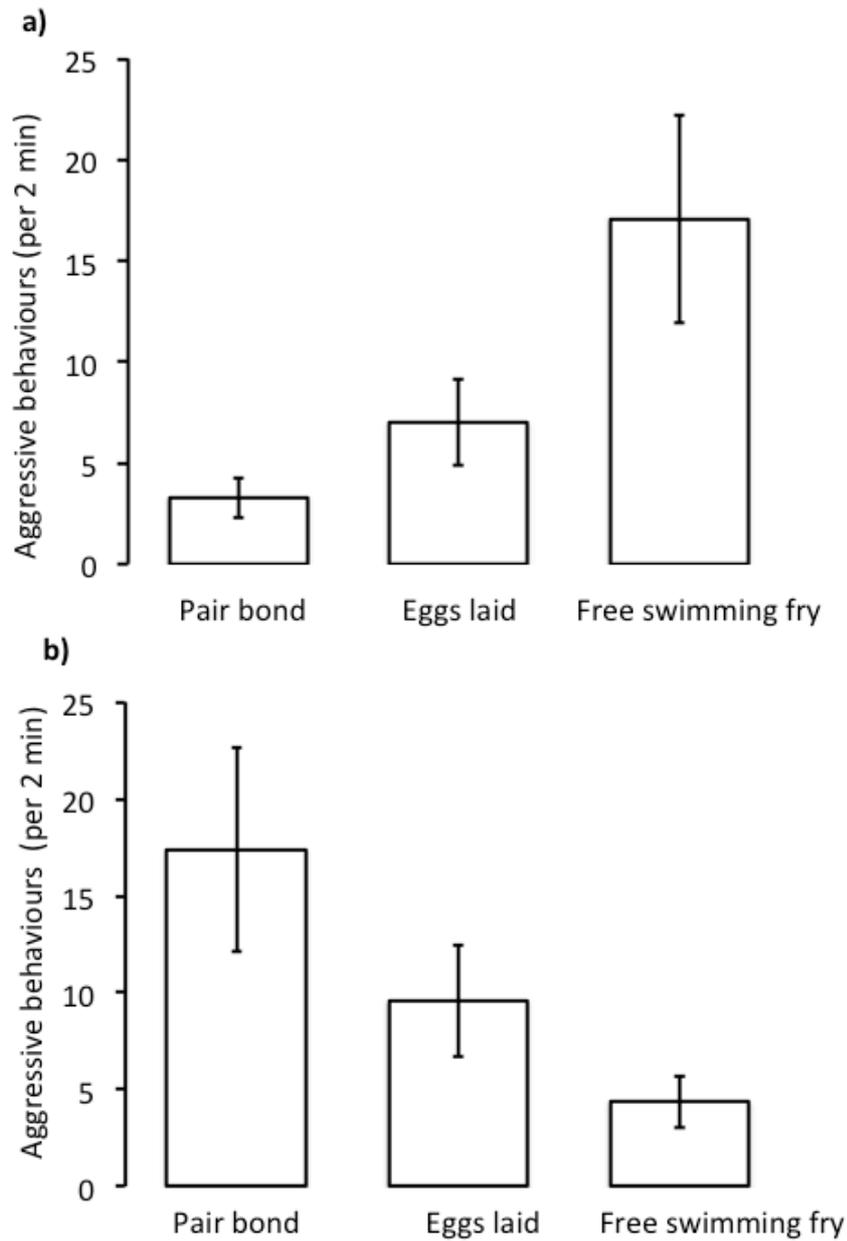
What about males? Although the above-mentioned selection pressures should equally affect males, we nevertheless found an opposite pattern. That is, we found that males were most aggressive towards model intruders when the pair had first formed and then reduced their level of investment in aggressive defence as the breeding cycle progressed. A commonly observed behaviour in many socially monogamous species is mate guarding (Komdeur et al., 1999; Saino et al., 1999; Chuang-Dobbs et al., 2001), where males actively guard females from sexual rivals. We consider the possibility that male *A. labiatus* may be mate-guarding females from conspecific competitors early in the breeding cycle. However, since Midas cichlids occupy territories for breeding purposes only and competition for these territories can be intense, any protection of the territory by males, even early in the breeding period, will also benefit the future survival of offspring. Another commonly observed behaviour in monogamous taxa is the desertion of mates and offspring by males (Keenleyside, 1983; Keenleyside, 1991; Amat et al., 2000). In many *Amphilophus* and related cichlid species, single females are frequently found occupying territories in the wild (Lehtonen et al., 2011a), and by the time young become independent, they are commonly guarded by only one parent (presumably the female; Barlow, 1976), suggesting that mate desertion is widespread in this group. Field studies conducted on other Neotropical cichlids have shown that if males abandon their brood prematurely, they usually do it only after offspring have become free swimming (Wisenden, 1994; Jennions & Polakow, 2001; Vélez et al., 2002). Desertion may also be more common in areas of high brood success and low predation levels (Townshend & Wootton, 1985), which may allow young to survive with only one parent (Wisenden, 1994). In our study, we observed that the territorial defence of males

was at its lowest during this free-swimming stage, suggesting that some males may be shifting their behaviour towards self-maintenance and possibly preparing for additional reproductive opportunities (Jennions & Telford, 2002).

To conclude, the results of our experimental stimulus manipulations showed a significant change in parental investment in aggressive defence during the progression of the breeding cycle. In particular, by controlling the appearance of intruders and timing of their presentation, we found that, at key stages, territorial defence was not shared equally. In this regard, it is possible that the sexes may be reacting to the conflict over care by investing more in territorial defence when it is most profitable for them to do so. Together, our results show that parental behaviour may not only vary between the sexes, but can also shift dramatically over the course of the breeding period.

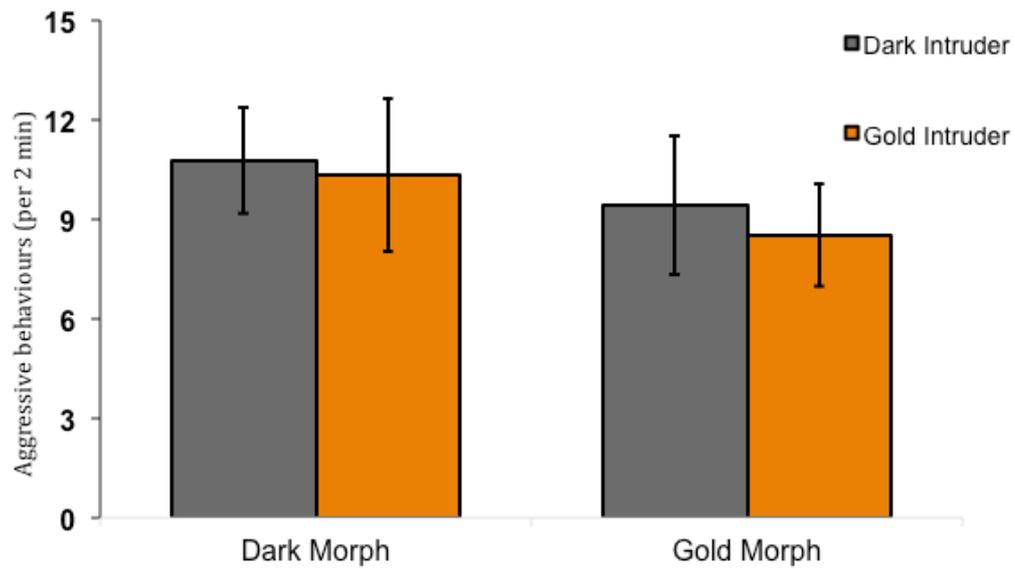
## Figures

**Figure 1**



**Fig 1.** The rate of aggression displayed towards model intruders by a) females and b) males at three key stages in the reproductive cycle ( $n = 11$  pairs).

Whiskers indicate standard error.

**Figure 2**

**Fig. 2** The average rate of aggression (on the y-axis) displayed by dark and gold territory holders (x-axis) towards both dark or gold intruder models. Whiskers indicate standard error.

**Supplementary Figure 1.** A dummy model, made from a photograph of a red devil cichlid, used to simulate a breeding territory intruder.



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## Declaration for Thesis Chapter

Monash University

## Declaration for Thesis Chapter Five

### Declaration by candidate

In the case of Chapter Five, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conceived and designed study, Conducted fish collection and behavioural experiments, Analysed data, Wrote manuscript	85%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Bob Wong	Helped conceive and design study, jointly conducted fieldwork, proof read manuscript	
Topi Lehtonen	Aided with statistical analysis, proof read manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work\*.

Candidate's Signature		Date
		25/11/2015

Main Supervisor's Signature		Date
		25/11/2015

# Chapter Five: Heterospecific intruder recognition, mate desertion and sex specific aggression in a biparental cichlid fish

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**Submitted manuscript**



*Crater Lake Xiloá, Nicaragua*

## Abstract

Despite obvious fitness benefits, offspring defence is likely to entail important costs to parents, such as energy loss and injury. To minimise these costs, territory holders should readily adjust their aggressive responses, according to the level of threat posed by different territory intruders. However, few studies have tested this ability in environments where an array of different heterospecific intruders frequently enter breeding territories. Accordingly, using a biparental cichlid fish (*Hypsophrys nematopus*) in crater Lake Xiloá (Nicaragua), we conducted an in-situ experimental manipulation, which assessed the ability of breeding pairs to respond aggressively to common territory intruder species that posed different levels of threat to their broods. In addition, we also observed breeding pairs and solitary females, to investigate parental investment and the consequences of mate loss for territory defence. We found, that breeding pairs can readily discriminate between different heterospecific intruders even in environments with a high frequency of territorial incursion, suggesting that *H. nematopus* have extensive behavioural flexibility in their aggressive response towards intruders. Furthermore, we show that females invest significantly more into territory defence than their male partners. However, while solitary females adjust the number of defensive behaviours they perform, they can not completely compensate for the loss of their partner, as intruders remain closer to their broods, than the broods of a breeding pair.

**Keywords:** Aggression, parental care, cichlid, heterospecific recognition, mate desertion

## Introduction

In species with parental care, the defence of offspring from predators often plays a key role in offspring success and consequently has important fitness consequences (Ridley, 1978, Perrone & Zaret, 1979, Clutton-Brock, 1991). Indeed, broods of parents that defend their offspring vigorously generally suffer a lower rate of predation (Greig-Smith, 1980, Tryjanowski & Golawski) and enjoy a higher recruitment rate (Kontiainen et al., 2009) than broods of less vigorous defenders. For example, White's skinks (*Egernia whitii*) that aggressively defend their territories from intruders also enjoy a higher offspring survival rate (Sinn et al., 2008). Similarly, more aggressive red-backed shrikes (*Lanius collurio*) have better reproductive success than less aggressive individuals (Tryjanowski & Golawski, 2004), highlighting the benefits of offspring defence.

Benefits aside, defending a breeding territory and offspring from intruders can also incur costs, such as energy loss and an increased risk of injury or mortality (Marler & Moore, 1988, Haller, 1996, Lappin & Husak, 2005, Requena et al., 2012). To reduce these costs, territory holders should have well-developed mechanisms for assessing the risk posed to their offspring by different types of intruders encroaching on their breeding territories (Swaisgood et al., 2004; Lehtonen et al., 2015; Lehtonen, 2014; Grether, 2011). For instance, female ground squirrels (*Spermophilus beecheyi*) assess the body temperature and size of Pacific rattlesnakes (*Crotalus viridis oreganus*) to evaluate the danger posed by individual predators to their offspring (Swaisgood et al., 2004).

However, surprisingly few investigations on intruder recognition or discrimination ability have been conducted in environments where an array of different heterospecific intruders may frequently invade the territory.

In biparental species, parental investment is often not shared equally and can become a source of conflict (Trivers, 1972, Wynne-Edwards, 1995). In particular, such a conflict may be based on differences in the potential reproductive rate of the sexes (Baylis, 1981, Reynolds, 1996) or a lack of assurance over paternity (Trivers, 1972, Keenleyside, 1991, Neff, 2003). To date, most studies of biparental behaviours have focused on the relative investment of the sexes in the context of offspring provisioning (Harrison et al., 2009). For example, Sanz and colleagues (2000) demonstrated that male, but not female, great tits (*Parus major*) reduce their offspring provisioning rate when their wings have been clipped to make flying more difficult. Similarly, non-handicapped females fully compensate for a handicapped male partner, whereas non-handicapped males will often decrease their feeding rate rather than compensate for their handicapped partner (Sanz et al., 2000). Yet, in contrast to what we know about sex differences in offspring provisioning, less attention has been given to the role of the sexes in territorial defence of offspring (Clutton-Brock, 1991, Clutton-Brock & Vincent, 1991, Székely & Cuthill, 1999, Harrison et al., 2009, Trnka & Grim, 2012). This is true, despite the potentially high fitness costs and benefits associated with the aggressive defence of offspring.

Mate and offspring desertion is common in many biparental species (Keenleyside, 1983, Keenleyside, 1991, Amat et al., 2000), and it is males who do it more often than females (McNamara et al., 2002). Although deserted females often stay and continue to care for their offspring in many (predominantly) biparental taxa (Trivers, 1972, Keenleyside, 1991, Lehtonen et al., 2011a, McNamara et al., 2002), the current knowledge of female behavioural compensations (if any) is based mostly on avian systems (for exception see Lehtonen et al., 2011b). Furthermore, previous studies investigating mate desertion

have typically focused on quantitative changes in care, with potentially important effects on the quality of care usually remaining unknown. For example, in avian studies, provisioning rates are often measured without any account of the quality of food being brought to the offspring (Marques, 2004, Harrison et al., 2009). Indeed, the main focus has been on quantitative behavioural adjustment also for taxa, in which brood defence is the main form of parental care provided to offspring (such as fish: Perrone & Zaret, 1979).

Accordingly, using a species of fish with biparental care, the poor man's tropheus cichlid (*Hypsophrys nematopus*), we conducted a field-based study to investigate sex differences in parental investment, the effects of mate desertion on single parents, and the ability of pairs to respond to different types of territory intruders. To this end, we first observed breeding pairs in order to assess sex differences in territory defence effort. We then compared the ability of pairs to defend offspring/territory compared with naturally occurring single parents. Second, we carried out an experimental study in the field to assess the ability of breeding pairs to recognize and adjust their aggression towards regular breeding territory intruders.

## Methods

### ***Study species and site***

*Hypsophrys nematopus* is a small monogamous substratum-spawning cichlid that is native to Central America, including Lake Xiloá in Nicaragua (McKaye, 1986, McKaye et al. 2010), where our study was carried out in January 2014. *Hypsophrys nematopus* is abundant in this volcanic crater lake and its continuous breeding season and stationary

breeding territories, along with the Lake's clear water, make the species an ideal candidate for in-situ behavioural observations (McKaye, 1986). We identified breeding pairs and single parent *H. nematopus* by their distinct breeding colouration (vertical white stripe on dark background) and their territorial behaviour amongst rocky shoreline habitat in the lake.

*Hypsophrys nematopus* coexists with several other small to medium sized fish species in Lake Xiloá (McKaye, 1977; authors' personal observations). The species co-occurring with *H. nematopus* vary widely in their ecological niche and breeding strategies and, as such, represent very different threats to the young and breeding territories of *H. nematopus*. *Hypsophrys nematopus* offspring rely on their parents to diligently protect them from brood predators (McKaye, 1977), as predation pressure and brood failure are high, with only a small percentage of young surviving to sexual maturity (McKaye et al., 2010). With such a high frequency of territorial incursions by heterospecifics, the need to accurately discriminate between many different individuals and recognise the threat they pose to the brood is likely to be critical to both parental and offspring fitness.

We carried out two separate studies to assess sex specific roles in brood defence, the consequence of mate desertion on brood defence, and the ability of pairs to discriminate between heterospecific intruders. First, we conducted observations of parental behaviours directed towards natural intruders in the wild. Second, based on the results of the observational study, we then carried out an investigation of interspecific intruder recognition by experimentally presenting pairs of *H. nematopus* with different types of heterospecific intruders differing in the threat they pose to offspring.

***Territory defence in breeding pairs and single parents***

We first observed *H. nematopus* pairs to investigate how parental investment, in regard to offspring and territory defence, is shared between the sexes in breeding pairs. To do this, we directly observed *H. nematopus* breeding territories that were occupied by either a cooperative breeding pair ( $n=36$ ) or a single parent ( $n=9$ ). In all cases, the latter was a solitary female. All observed *H. nematopus* had a brood of free swimming fry, to control for any sex differences in behaviour that may occur at different stages throughout the brood cycle. Breeding territories were observed at a distant vantage point, or if required, with a snorkel. After a 5-minute acclimation period, we recorded the brood defence behaviours of the pair or single female, toward naturally occurring territory intruders, for 10 minutes. All territory intruders were identified to species level, or if this was not possible, the lowest possible taxonomic level. Each individual act of aggression by a parent fish was classified according to one of two categories: 'attacks' (rapid movement towards the intruder until it fled) and 'chase' (an attack that was continued even after the intruder had started to flee; as per Lehtonen et al., 2010). We also measured the reaction distance, that is, the distance between the intruder and the centre of the brood when the parent initiated an aggressive behaviour. Furthermore for chase behaviour, we measured the distance between where the chase was initiated by the parent to where the parent stopped chasing the intruder. A 50cm measuring tape was placed on the substrate outside the breeding territory to provide a reference distance scale.

Using the above data, we were able to assess the total number of aggressive behaviours (attacks and chases) and also a measure of the quality of territory defence (reaction and chase distances of *H. nematopus*). After the observation trial, the total lengths of the

male and female were estimated, as were the number of fry and the median total length of fry in the brood (see Lehtonen et al., 2010, Lehtonen, 2011b). After the observation period was completed, a stone was marked in the territory to prevent assessing each pair or individual more than once.

We had three response variables, the total number (i.e. count) of aggressive behaviours per observation period, the reaction distance and the distance intruders were chased by *H. nematopus*, which we analysed using separate generalized mixed models, each with a negative binomial error distribution (as appropriate for overdispersed count data).

Three models assessed the effects of sex (male or female pair member), the number of fry and the median length of fry on the response variables respectively. The other three models assessed the effects of parent number (single or pair), the number of fry and the median length of fry on the three response variables (i.e. number of aggressive behaviours, reaction distance and chase distance). In each model, territory ID was added as a random effect to account for non-independence of the actions of a male and female defending a territory. We then simplified each of the six models in a stepwise fashion by assessing whether we could refit the model without the least significant term of the highest remaining order. We applied  $\chi^2$ -tests (with  $p = 0.05$  as the cut-off point) for this purpose. We used R 3.0.0 software (R Development Core Team) for all statistical analyses.

#### ***Experimental exposure to regular territory intruders***

Next, we tested the ability of *H. nematopus* pairs to distinguish between species that regularly encroached on their breeding territories. Specifically, we compared the aggressive responses of *H. nematopus* pairs to three species that we observed regularly

encroaching on breeding territories during the above observational study, that is, convict cichlids (*Amatitlania siquia*), poeciliids (*Poecilia sphenops*) and bigmouth sleepers (*Gobiomorus dormitor*). These species are expected to represent different levels of threat to *H. nematopus* young and breeding territories. Specifically, *G. dormitor* is an ambush predator and a specialised predator of cichlid fry and small juveniles (Bedarf et al., 2001), *A. siquia* is a breeding site competitor and an opportunistic fry predator (authors' personal observations), while the herbivorous *P. sphenops* poses little or no risk to breeding territories and fry.

We presented breeding pairs, that had a brood of free-swimming fry, with these intruder stimuli to test specifically whether, (1) *H. nematopus* breeding pairs could recognise the risk posed by different territory intruders and were discriminate in their level of aggressive behaviour, and also, (2) if *H. nematopus* breeding pairs differ in their latency to attack intruders depending on the level of threat they pose. We also investigated the influence of the number and size (total length) of fry on the total number of aggressive behaviours and the latency to attack intruders. To do this, we haphazardly collected representatives of our intruder stimulus species from the shallows of Lake Xiloá and presented them to *H. nematopus* breeding pairs, one at a time, in a cylindrical glass container (17cm high, 7.5cm circumference ; *sensu* van Breukelen, 2015). All intruder species were sized matched to control for biases in aggression by *H. nematopus* towards different sized intruders (*A. siquia* mean total length 5.5cm, *P. sphenops* mean total length 5.3cm, *G. dormitory* mean total length 5.6cm) Each *H. nematopus* pair ( $n=24$ ) was exposed to all three intruder species and a control (an empty glass container), in a randomized and unique presentation order. Each intruder was placed within the reaction distance of the territory-guarding parents (30cm from the brood centre). Pairs

were exposed to each intruder for two minutes. During that time we noted the latency to attack (charges and/or bites) and counted the total number of aggressive behaviours directed towards the intruder by both pair members. We gave pairs a break of five minutes between subsequent intruder presentations. After a pair had been presented with all three intruders and the control, we estimated the total lengths of the male and female and the median length and total number of fry in the brood and released intruder fish at the point of collection.

We had two response variables, the total number (i.e. count) of aggressive responses per observation period and the latency to attack (i.e. count of seconds before first attack), which were analysed in separate generalized mixed models with a negative binomial error distribution (as appropriate for overdispersed count data). In both models, we assessed the influence of intruder species, brood size (i.e. fry number) and median fry length by assigning these variables as fixed factors. To account for non-independence of the actions of a male and female defending a territory, territory ID was added as a random effect in both models. We then simplified each model in a stepwise fashion by assessing whether we could refit the model without the least significant term of the highest remaining order. We applied  $\chi^2$ -tests (with  $p = 0.05$  as the cut-off point) for this purpose. We used R 3.0.0 software (R Development Core Team) for all statistical analyses.

## Results

### *Territory defence in breeding pairs and single parents*

When applying a generalized mixed model to assess the total number of aggressive

behaviours (in regard to sex, fry number and fry length) all interactions were non-significant (in all cases  $p > 0.05$ ) and were hence removed from the initial model.

However, we did find a significant effect of sex, with females attacking intruders significantly more than males ( $z_1 = -5.63$ ,  $p < 0.0001$ , Fig. 1). Applying a generalized mixed model to assess the total number of aggressive behaviours (in regard to parent number, fry number and fry length) we found all interactions were non-significant ( $p > 0.1$ ) and hence removed from the initial model. However, we did see a significant effect of fry number, with both males and females being more aggressive when they had larger broods of fry ( $z_1 = 3$ ,  $p = 0.03$ , Fig. 2).

When we applied a generalized mixed model to assess reaction distances (in regard to sex, fry number and fry length) we found all interactions were non-significant ( $p > 0.10$ ) and hence removed from the initial model. We also did not find any effects of sex ( $z_1 = 1.79$ ,  $p = 0.82$ ), the number ( $z_1 = 1.79$ ,  $p = 0.07$ ) or the length of fry ( $z_1 = -0.13$ ,  $p = 0.89$ ). Applying a generalized mixed model to assess the reaction distance (in regard to parent number, fry number and fry length) we found all interactions were non-significant ( $p > 0.05$ ) and hence removed from the initial model. The final model with main effects indicated a marginally non-significant effect of pair type, ( $z_1 = 3.06$ ,  $p = 0.06$ ) and a significant effect of fry number ( $z_1 = 2.09$ ,  $p = 0.001$ ), with larger broods eliciting more aggressive behaviours.

When we applied a generalized mixed model to assess chase distances (in regard to sex, fry number and fry length) we found all interactions were non-significant ( $p > 0.10$ ) and hence removed from the initial model. We also did not find any effects of sex ( $z_1 = 1.89$ ,  $p = 0.52$ ), the number ( $z_1 = 1.41$ ,  $p = 0.08$ ) or the length of fry ( $z_1 = -0.16$ ,  $p = 0.54$ ). Applying a

generalized mixed model to assess chase distances (in regard to parent number, fry number and fry length) we found all interactions were non-significant ( $p > 0.10$ ) and hence removed from the initial model. We did find a significant effect of parent number with pairs having a greater chase distance than single females ( $z_1 = 1.55$ ,  $p = 0.03$ , Fig 3).

#### **Experimental exposure to regular territorially intruders**

When applying a generalized mixed model to assess the total number of aggressive behaviours (in regard to intruder species, fry number and fry length) we found a significant interaction between the number of fry and the median length of fry ( $z_2 = -0.01$ ,  $p = 0.044$ ). That is, the aggressive response of *H. nematopus* pairs towards intruders increased with fry number while simultaneously decreased with fry length, with parents more actively defended large groups of small fry. We also found a significant effect of intruder species. Specifically, *A. siquia* were attacked significantly more often than other intruder species ( $z_1 = -5.61$ ,  $p < 0.0001$ , Fig. 4). We also found a significant effect of fry number ( $z_1 = 2.88$ ,  $p = 0.003$ ), with pairs attacking intruders more vigorously when they had a large number of fry.

Applying a generalized mixed model to assess the latency to attack (in regard to intruder species, fry number and fry length) we found all interactions were non-significant ( $p > 0.05$ ) and hence removed from the initial model. We did find a significant effect of intruder species. That is, pairs were significantly slower to attack *P. sphenops* than other intruder species ( $z_1 = 2.16$ ,  $p = 0.031$ , Fig. 5).

## Discussion

We observed that females had a higher rate of aggressive behaviour and therefore invested significantly more in territory defence than their male partners. Naturally occurring single females adjusted their level of aggressive territory defence. However, single females could not fully compensate for the loss of their partner, with single females chasing intruders significantly less distance from their broods in comparison to pairs. When presented with commonly encountered intruder species, we found that *H. nematopus* breeding pairs were discriminate in their aggressive responses. Pairs demonstrated that they could readily distinguish between intruder species that were likely to pose different levels of threat to their offspring and/or breeding territory. Specifically, pairs were significantly more aggressive to the presence of *A. siquia* in their breeding territory than to *G. dormitor*, while *P. sphenops* elicited the most delayed and least aggressive response. Lastly, in both our experiment and observational studies, the number of fry in a brood significantly influenced how vigorously a breeding pair and single female defended their territories from intruders, with larger broods eliciting a greater aggressive response.

The level of investment placed into territory defence was not shared equally between the sexes. Specifically, we observed that females were significantly more aggressive (i.e. had a higher total count of aggressive responses) towards territory intruders than males. This is in contrast to other previous studies that have investigated sex specific parental roles in biparental cichlids and found that males perform territory and/or brood defence at a higher rate than females (Keenleyside & Bietz, 1981, Itzkowitz, 1984, Nakano & Nagoshi, 1990, Stiassny & Gerstner, 1992). However, it has also been noted that female participation in territory defence does often increase as the brood cycle

progresses (Rogers, 1988, Murry et al., 2001, Itzkowitz et al., 2005, McKaye & Murray, 2008), for example, when fry become free swimming, which is the stage at which we observed *H. nematopus* breeding pairs.

Previous studies, mostly on birds, have shown that although females adjust their workload following decreased male participation, they are unable to completely compensate for the absence of a partner (Houston et al., 2005, Harrison et al., 2009), as seen for example in the Spanish sparrow (*Passer hispaniolensis*; Marques, 2004). Here, we found that single females exhibited a higher number of aggressive behaviours towards territory intruders than paired females. However, we found that single females did not (or could not) chase territory intruders as far away from their broods, compared to pairs. Consequently, territory intruders remained closer to the brood of single females than they did to a cooperative pair. The quality of territory defence that a single female can provide to her offspring is therefore likely to be lower than a pair and may lead to reduced reproductive success. This is concordant with Lehtonen et al. (2011b), who found that solitary female *Amphilophus sagittae* and *Amphilophus zalius* cichlids adjust their rate of aggressive responses in the absence of a partner, but similarly cannot provide the same quality of care as a cooperative pair. Hence, our findings underscore the importance of considering both the quality, as well as the total number of, parental behaviours (Harrison et al., 2009, Lehtonen et al., 2011b).

We found that *H. nematopus* pairs were able to discriminate between different species of common territory intruders. Specifically, pairs reacted more aggressively when an *A. siquia* was present in their breeding territory as compared to the two other intruder species. *Hypsophrys nematopus* and *A. siquia* have very similar breeding patterns,

reproductive behaviours and peak breeding periods (McKaye, 1977, McKaye et al., 2010). Both species are limited by breeding space, particularly in Lake Xiloá (McKaye, 1977) and intense interspecific competition is likely to exist between them.

Furthermore, *A. siquia*, like many other cichlid species, will opportunistically consume the eggs and fry of other fish and are therefore a direct threat to *H. nematopus* offspring (Mackereth & Keenleyside, 1993).

Surprisingly, we did not see more aggression directed towards *G. dormitor*, which are specialised predators of fish fry and juveniles. For example, a previous observation of *A. siquia* showed that breeding pairs are more aggressive towards *G. dormitor* in comparison to other species (Alonzo et al., 2001). *Gobiomorus. dormitor* is a cryptic species, which generally positions itself motionlessly before rapidly striking out at broods (Alonzo et al., 2001). This behaviour is likely to have evolved to allow *G. dormitor* to get as close to broods as possible and may have made it less observable to *H. nematopus* than other species in our experimental set-up. Finally, we found that *P. sphenops* elicited the slowest and least aggressive response. This is concurrent with a previous observational study, which noted that poeciliid species are tolerated closer to the broods of breeding cichlids than other species (Wisenden et al., 2015).

*Hypsophrys nematopus* must be subject to selection for extensive flexibility in their behavioural responses, in order to respond readily to different heterospecific intruders. This flexibility is likely to be crucial for reproductive success, particularly in Lake Xiloá and similar environments, where many species compete for breeding space and there is a high incidence of territorial incursions, brood failure and intense predation pressure

(McKaye, 1977). Moreover, by recognizing and not responding to species that pose a low threat, territory holders can minimize costly aggressive behaviours. Some bird species can also discriminate between low and high risk intruders. For example, nesting red-backed shrikes (*Lanius collurio*) and barred warblers (*Sylvia nisoria*) can readily differentiate between a predator and non-predator model (Polak, 2013). Similarly, in the African cooperative breeding cichlid, *Neolamprologus pulcher*, group members can readily identify different territory intruders and adjust their defence behaviours according to their own social status (Desjardins et al. 2008). Whereas, other Neotropical cichlid species (*Amphilophus sp.*) are highly attuned to the breeding status of intruding heterospecific congeners, with intruders that display breeding colouration presenting less threat to broods and receiving significantly less aggression by territory holders (Lehtonen et al., 2015, Lehtonen et al., 2010). Together with our study, these results suggest that intruder recognition is an important trait, which is potentially widespread among offspring guarding Neotropical cichlids.

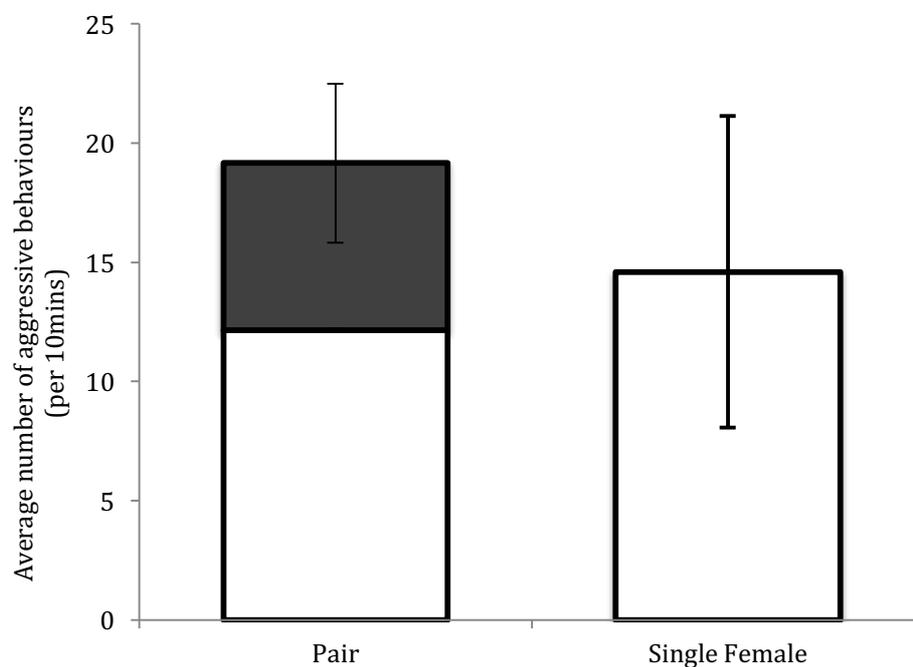
Lastly, we found that offspring number was positively associated with aggressive behaviours and shorter reaction distances. In environments with high predation pressure and brood failure, one would expect parents to adjust their level of territory defence according to the value of the brood at stake (Trivers, 1972, Clutton-Brock, 1991). Indeed, previous studies have predicted that larger broods are more valuable to parents and should be defended more vigorously than smaller broods (Andersson et al., 1980, Greig-Smith, 1980, Montgomerie & Weatherhead, 1988, Redondo, 1989). For example in birds, both the Imperial Shag (*Phalacrocorax atriceps*) and the redwing blackbird (*Agelaius phoeniceus*) invest more into nest defence as brood size increases

(Robertson & Biermann, 1979, Svagelj et al., 2012). Similarly, in cichlids, fry number has previously proven to be an important determinant of parental investment in territory defence. For instance, female *Aequidens coeruleopunctatus* adjust their parental behaviour in response to changes in brood size, that is, females with experimentally reduced broods are easier to scare away when threatened and stay away for longer than females with experimentally augmented broods (Carlisle, 1985), suggesting that brood size influences parental behaviours across taxa.

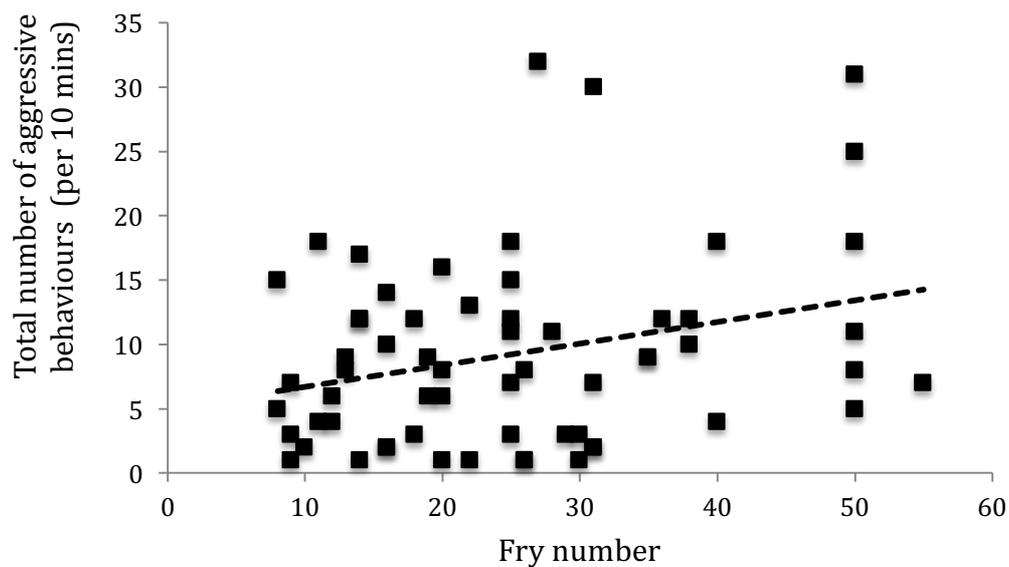
In conclusion, we found that *H. nematopus* readily differentiates between commonly encountered species encroaching on its breeding territory. This flexibility of behaviour in terms of recognising species that pose different threat levels is likely to be critical for reproductive success and minimising costly aggressive behaviours, particularly in environments like Lake Xiloá, with high brood failure and predation. In a breeding pair, females invested significantly more into territory and offspring defence than males. If females were abandoned, they responded by increasing the number of aggressive territorial behaviours they performed. However, single females could not completely compensate for the loss of their partner, as intruders remained closer to their broods, than the broods of breeding pairs.

## Tables and Figures

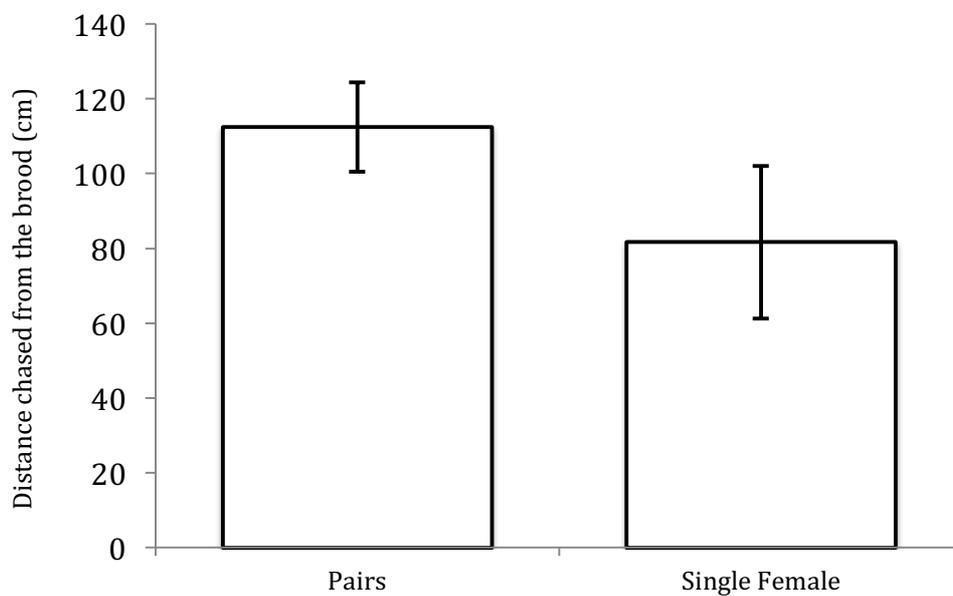
**Figure 1**



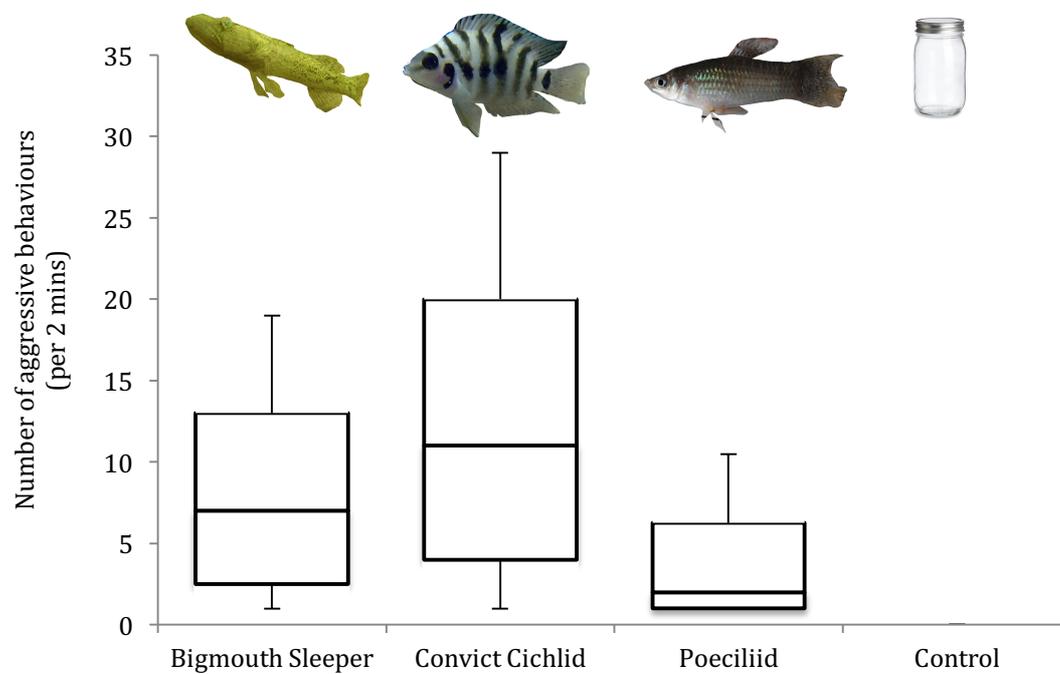
**Fig 1.** The number of aggressive behaviours directed towards territory intruders by pairs and single females. White = female, grey = male. Error bars indicate s.e. We found that pairs (left) displayed significantly more aggressive behaviours than single females (right). However, within a pair (left) females (white) displayed significantly more aggressive behaviours than their male partners (grey).

**Figure 2**

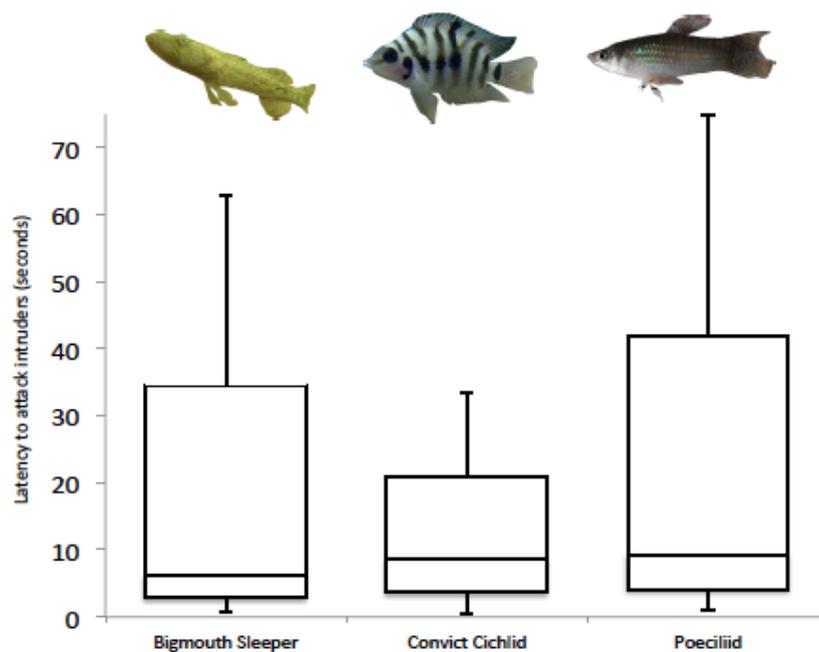
**Fig. 2.** The relationship between fry number and number of aggressive behaviours directed towards territorial intruders. Error bars indicate s.e.

**Figure 3****Fig. 3.** The distance pairs chased territory intruders in comparison to single females.

Error bars indicate s.e.

**Figure 4****Fig. 4.** The number of aggressive behaviours directed towards territory intruders.

Central horizontal lines within the boxes, margins of the boxes and whiskers indicate means, s.e. and s.d., respectively. Photos, Topi K. Lehtonen.

**Figure 5**

**Fig. 5.** The latency to attack territory intruders. Central horizontal lines within the boxes, margins of the boxes and whiskers indicate means, s.e. and s.d.

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## Chapter Six: Discussion

To summarise, using a multi-disciplinary approach, I found significant variation in the phenotypic response of cichlid individuals to a novel environment, clear behavioural and morphological variation between colour morphs, and significant sex differences in the level and timing of parental investment. Together, these results not only demonstrate the high degree of variation within Neotropical cichlid populations, but also provide insights into both the generation and maintenance of intraspecific variation, which in turn is important for understanding how populations diversify and, ultimately, how new species form.

In Chapter 2, I showed that a recently introduced red devil population is phenotypically variable in regard to lip size, a trait that appears to be associated with diet. Specifically, as lip size increased, so did the proportion of algae in the diet. By contrast, decreasing lip size was associated with a decrease in the proportion of insects in the diet. These findings suggest that this population may be showing the initial signs of diversification, with variation in a key trophic trait, hypertrophied lips, being used to exploit different resources in a novel environment. More generally, the result highlights how rapidly Neotropical *Amphilophus* cichlids can phenotypically respond to their environment after a recent colonization event. Elsewhere, for example in Lake Apoyeque, Nicaragua, a large-lipped *Amphilophus* phenotype has evolved from a small-lipped form within historical times (~100 years; Elmer et al. 2010), while in other nearby crater lakes exploitation of vacant ecological niches is a primary hypothesis explaining the rapid speciation of endemic Midas cichlids (Barluenga et al. 2006). However, in a closely

related Neotropical cichlid *Herichthys minckleyi*, a high degree of trophic differentiation between individuals has not resulted in any genetic differentiation between the ecotypes (Kornfield & Koehn 1975). Therefore, it is difficult to make any clear predictions about the future direction and extent of diversification that will occur in the introduced Hazelwood red devil population. Experimentally, I did not find that this phenotypic variation in red devils reflected any differences in feeding performance on different substrate complexities or diet type treatments. Similarly, I did not observe any plastic response in lip size or head shape in response to different food hardness or substrate complexities during the development of red devil juveniles. Compared to individuals in the native range, the lip size of introduced red devils is smaller and the latter population has significantly lower genetic diversity. Notably, such morphology-dependent specialisation has taken place despite reduced standing genetic variation in the population.

I found that dark morph red devil individuals, which are more abundant in natural populations, were able to adjust the shade of their body colouration between substrates, with individuals being brighter on light coloured substrate and darker on dark substrate (Chapter 3). Gold individuals, by contrast, did not (or could not) alter their body to match their background substrate. Therefore, despite well-documented intraspecific benefits to being gold (Barlow, 1983) and the dominant inheritance of the gold morph (Maan & Sefc, 2013), gold individuals are potentially disadvantaged by being more conspicuous to visual predators. Consequently, differences in the ability to background match could play a potentially important role in maintaining colour morph frequencies.

Within breeding pairs, I found that red devil males and females varied significantly in the level of parental investment they provided toward territory defence over the course of the breeding cycle (Chapter 4). In particular, females were most aggressive towards territorial intruders later in the breeding cycle, when fry were free swimming and potentially more vulnerable to predators. In contrast, the level of territorial defence displayed by males was highest when the pair had first formed and subsequently tapered with time. This variation in parental investment suggests that the prioritise of the sexes differs at key stages in the brood cycle, and that males and females are investing more in territorial defence when it is most profitable for them to do so. Moreover, in the current study, we found no evidence that territorial aggression was significantly affected by the colour of the intruder or parent. However, previous studies have found body colour to be important in influencing aggressive behaviour in other closely related species (Lehtonen, 2014, Lehtonen et al., 2015).

Similar sex specific roles were also observed in another Neotropical species, *H. nematopus* (Chapter 5). Specifically, I found that females invested significantly more in territory defence than their male partners. Naturally occurring single females adjusted their level of aggressive territory defence to compensate for the lack of a male partner. However, single females could not fully compensate for the loss of their partner, with single females being unable to chase intruders as far away from their brood compared to paired females. When presented with commonly encountered heterospecific intruder species, breeding pairs were found to be highly discriminating in their aggressive responses. Pairs demonstrated that they could readily distinguish between intruder species that posed different levels of threat to their offspring and/or breeding territory. This ability to recognise species that pose different threat levels is likely to be critical for

reproductive success and minimising costly aggressive behaviours, particularly in environments with high brood failure and predation. These results suggest that selection for behavioural flexibility in response to different intruders is likely to be strong and demonstrates the importance of assessing not only the relative number of parental behaviours but also the underlying quality of parental behaviors.

In conclusion, my research highlights intraspecific variation in key morphological and behavioural traits in Neotropical cichlids. Specifically, I found that within cichlid populations, individuals differ in regard to their trophic morphology, body colour and parental investment and that these differences are directly associated with differences in diet, background matching ability and sex. Together, the results of my thesis provide further insights into our understanding of intraspecific variation and contribute to the ongoing investigation into the origins and drivers of organismal diversity.

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# Appendix



*An Amphilophus breeding pair inspect a dummy model intruder in Lake Xiloá, Nicaragua.*

*Photo credit, Topi K. Lehtonen.*

# Cichlid Fish Use Coloration as a Cue to Assess the Threat Status of Heterospecific Intruders

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Submitted February 9, 2015; Accepted May 29, 2015; Electronically published August 11, 2015

Dryad data: <http://dx.doi.org/10.5061/dryad.r0h7s>.

**ABSTRACT:** The ability to assess the threat posed by competitors, and to respond appropriately, is important for reducing the costs of aggression. In this respect, aggression directed toward heterospecifics is often just as significant as aggression among conspecifics. This is especially true for cichlid fish that share breeding grounds with heterospecifics. Indeed, cichlids are known to differentiate not only between conspecifics that pose different levels of threat but also between heterospecific territorial intruders by directing more aggression toward nonbreeding individuals. To assess whether the ability to make such distinctions could be based on color cues alone, we carried out a field study in which we experimentally presented *Amphilophus sagittae* cichlid pairs with model intruders of a sympatric congener, *Amphilophus xiloaensis*, in breeding versus nonbreeding coloration. Consistent with our prediction, we found that *A. sagittae* exhibited more aggression toward *A. xiloaensis* models of the latter color type. The results are, to our knowledge, the first to show that territory holders can, based on coloration alone, assess variation among individuals of a species other than their own in the threat posed to offspring survival.

**Keywords:** color signal, competitor recognition, familiarity, heterospecific aggression, signal reliability, species interactions.

## Introduction

Aggressive behavior usually entails costs, such as the loss of energy (Haller 1995; Neat et al. 1998; Brandt 2003; Castro et al. 2006), time taken from other activities (Radesäter et al. 1987), and increased risk of injury (Neat et al. 1998; Lappin and Husak 2005) or predation (Marler and Moore 1988). Failing to react aggressively to a serious threat can also result in negative fitness consequences. We should therefore expect animals to have well-developed mechanisms for distinguishing among different types of opponents and adjusting their level of aggression accordingly (Grether 2011). Such

biases in aggressive behavior may have significant evolutionary consequences—not only within species but also in the context of heterospecific interactions (Seehausen and Schluter 2004). Indeed, according to recent evidence, aggressive interactions between species are very common and can be just as important as aggression among conspecifics (Ord and Stamps 2009; Peiman and Robinson 2010; Ord et al. 2011; Grether et al. 2013). Nevertheless, the evolutionary consequences of heterospecific aggression are rarely addressed and, hence, remain poorly understood (Grether et al. 2009, 2013; Peiman and Robinson 2010).

In the context of the strategic allocation of aggression, patterns of phylogenetic relatedness and phenotypic similarity among competitors are likely to be important. These factors may not only affect the fitness consequences of aggressive behavior but also how easy it is to differentiate between intruders that pose different levels of threat (Grether 2011; Grether et al. 2013). In this regard, a recent meta-analysis suggests that heterospecific aggression is more pronounced among congeneric heterospecific individuals, as compared to those from more distantly related genera (Peiman and Robinson 2010). One likely contributor to such a pattern is cognitive limitations; that is, aggression may simply be biased toward those heterospecific individuals that phenotypically resemble conspecifics (Peiman and Robinson 2010), and the same cognitive and sensory systems may be used in signal recognition toward both conspecific individuals and those of closely related, phenotypically similar species (Ord et al. 2011).

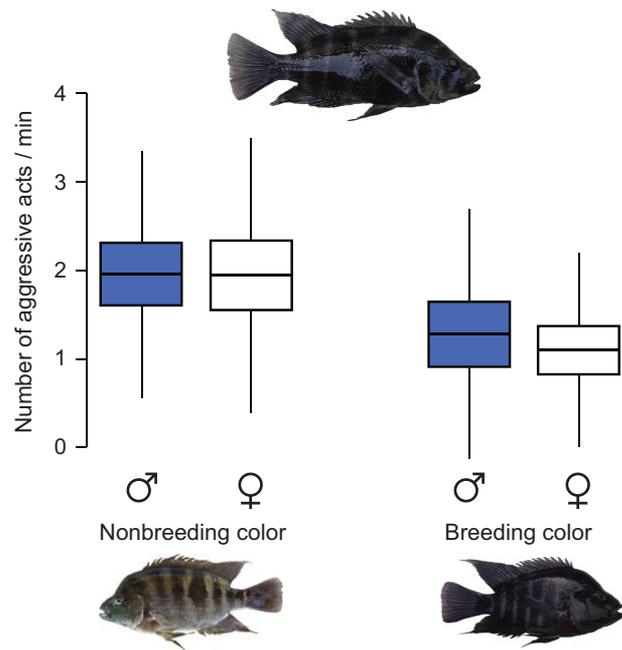
Even when competing species are phenotypically similar or congeneric, aggression is not necessarily symmetrical, implying that phenotypic similarity is not the only factor governing heterospecific aggression biases. For example, when two species of grey tree frogs are calling at the same breeding pond, male *Hyla versicolor* suffer a greater reduction in attractiveness due to call overlap than male *Hyla chrysoscelis* (Marshall et al. 2006; Reichert and Gerhardt 2014), with male *H. versicolor*, in turn, being more likely

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to initiate physical heterospecific aggression (Reichert and Gerhardt 2014). Furthermore, there is variation among taxa in the types of cues that are most often used for distinguishing among territorial intruders at close range (Grether 2011). In fish, color is known to play an important role in both species and competitor recognition (Barlow 1974; Endler 1983; Seehausen et al. 1999). For instance, in Lake Victoria cichlid fishes of the genus *Pundamilia*, the competitive advantage of red over blue males was diminished when encounters took place under green light conditions, which prevented the fish from seeing the color differences (Dijkstra et al. 2005). Similarly, *Pseudotropheus* cichlids from Lake Malawi directed more aggression in staged encounters toward members of a species with color patterns similar to their own than toward a differently colored, but more closely related, species (Pauers et al. 2008).

Neotropical cichlids are also known to exhibit biases in social interactions, including territorial aggression, depending on the color of their conspecific opponents (Barlow 1974, 1983; Lehtonen 2014). This is especially true for the Midas cichlid species complex (*Amphilophus* spp.; sensu Barluenga and Meyer 2010; Elmer et al. 2010; Geiger et al. 2010), which has been one of the prime systems for evolutionary studies, especially regarding parallel and sympatric speciation (Barluenga et al. 2006; Salzburger 2009; Elmer et al. 2010). Here, we focused on aggression during the breeding season, when the breeding grounds of these fish are typically densely occupied with multiple, simultaneously breeding species of cichlids (McKaye 1977; T. K. Lehtonen, personal observations). These breeding aggregations are characterized by intense competition for territory space among conspecific, congeneric, and more distantly related cichlids (McKaye 1977; T. K. Lehtonen, personal observations). When ready to spawn, each cichlid pair claims a sedentary breeding territory, the defense of which (and, later, also that of juveniles) is the most notable form of aggression within and between these species in the wild (McKaye 1977; Barlow 2000). Thus, territorial aggression is directed toward (i) competitors for territory space (both conspecific and heterospecific), (ii) brood predators (both conspecific and heterospecific), and (iii) conspecific sexual competitors (McKaye 1977; Lehtonen et al. 2010, 2012).

In this study, we tested the hypothesis that color alone is a sufficient cue for an adjustment of territorial aggression in *Amphilophus sagittae* toward a sympatric congener, *Amphilophus xiloaensis*, displaying either breeding or nonbreeding coloration. Of note, in the majority of individuals of both species (i.e., more than 80%; Elmer et al. 2009), breeding males and females are black, often with some brighter (mostly gray) markings, while nonbreeders of both sexes have a gray base coloration with dark vertical bars (fig. 1; Barlow 1974; Lehtonen et al. 2010). Furthermore, an earlier study conducted in Lake Apoyo, Nicaragua,



**Figure 1:** Total rate of aggression toward model opponents by male (blue boxes) and female (white boxes) *Amphilophus sagittae* territory holders. *Amphilophus sagittae* is pictured at the top of the graph. The model intruders were either nonbreeding (bottom left) or breeding (bottom right) colored *Amphilophus xiloaensis*. Central horizontal lines within the boxes indicate means, margins of the boxes show standard errors, and whiskers indicate standard deviations. The number of territories sampled is 15.

found that territory-holding *Amphilophus* cichlids directed more intense aggression toward both conspecific and heterospecific nonbreeders than toward breeders (Lehtonen et al. 2010). In the current study, we predict that coloration alone may be a sufficient cue for the adjustment of territorial aggression to the different heterospecific intruder types. We therefore expect territory holders to make a distinction between the two intruder types and adjust their behavior accordingly, even when the intruders differ only in their color markings.

### Material and Methods

This field-based study was conducted using scuba in Lake Xiloá, Nicaragua (lat. 12°12.8'N; long. 86°19.0'W), between December 2013 and January 2014, when all cichlid species in the lake (McKaye 1977) are breeding (Lehtonen et al. 2011; T. K. Lehtonen, personal observations). To investigate their aggressive responses to different types of opponents, we presented live *Amphilophus sagittae* breeding pairs ( $N = 15$ ; at a water depth of  $9.9 \pm 0.4$  m [mean  $\pm$  SE]) with dummy stimulus models of a sympatric competitor, *Amphilophus xiloaensis*, which is closely related and pheno-

typically quite similar to the focal species (fig. 1) but has a deeper body and more benthic lifestyle (Elmer et al. 2009). While *A. xiloaensis* breed, on average, in deeper water than *A. sagittae* and are more often found in association with rocky rather than mixed habitat (i.e., alternating patches of rocks and finer substratum), the two species are frequent territorial neighbors (Elmer et al. 2009; Lehtonen et al. 2011; T. K. Lehtonen, personal observations).

Handmade models have been successfully used as stimuli to elicit ecologically relevant behaviors in a range of fish species (reviewed in Rowland 1999), including *A. sagittae* (Lehtonen 2014). The use of models (or dummies) have a significant advantage over the use of live stimulus animals because they allow us to explicitly control for confounding factors that might otherwise arise from differences in the behavior of the stimulus animals. However, instead of using the more traditional wax or painted shapes employed by other researchers (Barlow and Siri 1994; Rowland 1999), we chose to use more realistic-looking models based on photographs of wild-caught fish following the methods of Lehtonen (2014). Specifically, stimulus models were made by gluing waterproof, photographic color prints of the lateral side of a live or freshly euthanized specimen onto both lateral sides of a fish-shaped floating plate (thickness = 6 mm). Each model was attached to a sinker with a thin, transparent fishing line, allowing it to float in a natural position approximately 15–20 cm above the lake bottom. All models were 16 cm long, a size that was easy to handle under water and that represents an overlap in size ranges of adult males and females in the wild. Half of the models were of *A. xiloaensis* cichlids in breeding color, while the rest were of nonbreeding individuals. We tested the reactions of territory-holding *A. sagittae* toward the two model types in a habitat characterized by pebbles lying on a finer substratum of sand and organic material.

Each replicate was initiated by placing an *A. xiloaensis* model, with either breeding or nonbreeding coloration (fig. 1; see also the general description above), at a distance of approximately 40 cm from the center of the focal *A. sagittae* territory. Typical aggressive responses involved either slow movement toward the model with flared gills and fins in a threat display or a rapid advance, often followed by a bite, before retreating back to the fry. We counted the total number of such aggressive encounters by both territory owners toward the model for 5 min, giving the total aggression rate (sensu Lehtonen et al. 2012; Lehtonen 2014). After a resting period of 5 min (during which the models were removed from sight), we repeated the procedure using the alternative model type (i.e., a model with breeding coloration if the pair had initially been presented with a model with nonbreeding coloration, and vice versa).

We had in total 14 different models of *A. xiloaensis*, half of them in breeding ( $N = 7$ ) and the other half in non-

breeding ( $N = 7$ ) coloration (with each model made using a photograph from a different fish). Following the approach of Stevens et al. (2007), to confirm that breeder and nonbreeder models were quantitatively different in their visual appearance, we assessed each model image's sum of the red, green, and blue scores (i.e.,  $R + G + B$ ) in ImageJ (U.S. National Institutes of Health, Bethesda, MD) using the six landmarks (here,  $50 \times 50$  pixels) described in Sowersby et al. (2015). We found that the nonbreeder models were, indeed, lighter colored (i.e., had a significantly higher  $(R + G + B)/3$  score) than breeder models (two-sample  $t$ -test,  $t_{12} < 3.4$ ,  $P < .01$  for all six landmarks). Hence, breeder and nonbreeder models differed from each other both in relation to their color markings and luminance. For clarity, from hereon, we refer to this visual dissimilarity between breeding and nonbreeding individuals as a difference in coloration. Because it was necessary to use individual models more than once, models of *A. xiloaensis* in breeding and nonbreeding coloration were paired haphazardly for the purpose of presentation to *A. sagittae*. As a result, one pairing of breeder and nonbreeder stimulus models ended up being used twice, whereas none of the other stimulus models were paired with the same alternate model more than once. The order of the model presentations was randomized (model with breeding coloration presented first in eight of the replicates; nonbreeding coloration presented first in the remaining seven), and a different *A. sagittae* territory was used for each replicate of the experiment.

#### Statistical Analyses

To assess the influences of sex (male vs. female) of the focal territory holders and the type of the model intruder (breeder vs. nonbreeder), we used R 3.1.0 software (R Development Core Team) to apply a generalized mixed model using the `glmmPQL` function of the packages `nlme` and `MASS`, with a negative binomial error distribution as appropriate for overdispersed count data (Zuur et al. 2013). To account for the nonindependence of the actions of a territory-holding male and female and the use of each model in more than one replicate, we added territory ID and model ID, respectively, as random factors (per the method described in Pinheiro and Bates 2000).

#### Results

When we applied a generalized mixed model to assess the effects of the sex of the focal territory holders and the color of the model intruder on the rate of aggression, we found no significant interaction between the two factors ( $t_{56} = 0.0227$ ,  $P = .98$ ). A model refitted without the interaction showed a significant effect of the intruder color ( $t_{57} = 2.60$ ,

$P = .012$ ) but not sex ( $t_{57} = 0.0648$ ,  $P = .95$ ): independent of their sex, *Amphilophus sagittae* territory holders were more aggressive toward nonbreeding than breeding colored *Amphilophus xiloaensis* models (fig. 1).

### Discussion

We found that *Amphilophus sagittae* territory holders directed more aggression toward model *Amphilophus xiloaensis* intruders that had nonbreeding coloration than toward those with breeding coloration. The result is concordant with an earlier study showing that *Amphilophus zaliosus* territory holders from Lake Apoyo were similarly more aggressive toward nonbreeding than breeding *Amphilophus astorquii* intruders (Lehtonen et al. 2010). However, in that study, behavioral responses toward heterospecifics were based on natural encounters. Therefore, it could not be established whether differences in the responses of territory holders were due to differences in the coloration of the heterospecific intruder or some other cue(s). In contrast, by using dummies to control for alternative cues such as intruder behavior or familiarity, we were able to show that *A. sagittae* adjust their aggression toward heterospecific intruders differing in breeding and nonbreeding coloration. More generally, the results of our study are also consistent with previous research in *Hetaerina* damselflies. In Anderson and Grether (2010), individuals were found to direct more aggression toward heterospecific species that have colors similar to their own. However, to our knowledge, the current study is the first to show that not only is coloration (here, differences in patterns and brightness) a sufficient cue for assessment of heterospecific territorial intruders at a species level but that territory holders can use such color-related cues to distinguish between individuals that differ in their potential threat even when these individuals are heterospecific.

For *A. sagittae* to adjust their aggressive responses based solely on coloration differences among *A. xiloaensis* intruders, territory holders need to be subject to selection for differential coloration-dependent aggression (ultimate explanation), in addition to having the ability to recognize individuals with different color patterns (proximate explanation). In regard to the former, an important driver of heterospecific aggression is thought to be the extent to which heterospecifics compete over the same resources, such as food, territories, shelter, and mates (see Genner et al. 1999; Dijkstra et al 2007). In this respect, even though neighboring breeding pairs occasionally engage in disputes over territory borders (T. K. Lehtonen, personal observations), individuals in breeding coloration are generally expected to pose a lower threat to territory owners than nonbreeders, especially in terms of offspring predation. This is because breeders already occupy a territory and are, them-

selves, preoccupied with caring for offspring; and since they are mostly relying on previously accumulated energy reserves during the parental phase, they are less inclined to predate on the fry of other cichlids (McKaye 1977; Rogers 1988; Barlow 2000; Lehtonen et al. 2010). Nonbreeding individuals are, by contrast, more likely to predate on the eggs and juveniles of both conspecifics and heterospecifics, and pose a threat through territorial takeovers. Our results, therefore, support previous findings showing that territory holders adjust their aggression accordingly (Lehtonen et al. 2010).

At the proximate level, because heterospecific aggression may occasionally also arise from misplaced conspecific aggression (Peiman and Robinson 2010), it is possible that *A. sagittae* territory holders responded to the differences in intruder coloration because they mistook them for conspecifics. This possibility would imply a selection pressure to differentiate between conspecific, but not heterospecific, breeders and nonbreeders. Such a possibility could arise because conspecific (but not heterospecific) nonpaired individuals can pose a significant sexual threat. If the models were perceived as a sexual threat, however, we would have expected male and female responses toward them to be asymmetric, which we did not observe. It also seems likely that if territory holders are able to correctly identify the breeding status of heterospecific intruders (even if not correctly identifying their species), adjustment of responses to the intruder status should be beneficial. Furthermore, although the two species have the capacity to hybridize, pairing in the wild is species assortative (Elmer et al. 2009), suggesting that species recognition is highly tuned and mistakes, if they occur at all, are rare. In this respect, even though courtship has been observed among members of the same species, we did not see any evidence of courtship among heterospecifics—either during this or earlier studies—thus supporting our interpretation that individuals are able to properly identify and distinguish heterospecifics from conspecifics.

To conclude, our results indicate that coloration alone is a sufficient cue for intruder status identification and that consequent adjustments of aggression are relevant even among heterospecific individuals.

### Acknowledgments

We are grateful for logistic support from M. Barluenga, L. and P. Lehtonen, K. and M. McKaye, and E. van den Berghe. We also thank K. Delhey and anonymous reviewers for their helpful suggestions to improve the manuscript. Funding was provided by the Department of Biology at the University of Turku, the Hoslworth Wildlife Endowment Fund, and the Linnean Society of New South Wales.

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Associate Editor: J. Albert C. Uy  
Editor: Susan Kalisz



Above, Lake Xiloá in Nicaragua. Below left and right, cichlid fish (*Amphilophus sagittae*) in Lake Xiloá. Photo credit: Topi K. Lehtonen.



## Research

**Cite this article:** Lehtonen TK, Sowersby W, Wong BBM. 2015 Heterospecific aggression bias towards a rarer colour morph. *Proc. R. Soc. B* **282**: 20151551.  
<http://dx.doi.org/10.1098/rspb.2015.1551>

Received: 28 June 2015

Accepted: 24 August 2015

**Subject Areas:**

behaviour, evolution, ecology

**Keywords:**

coexistence, colour polymorphism, fitness cost, heterospecific aggression, morph frequency, species interaction

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Electronic supplementary material is available at <http://dx.doi.org/10.1098/rspb.2015.1551> or via <http://rspb.royalsocietypublishing.org>.

# Heterospecific aggression bias towards a rarer colour morph

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Colour polymorphisms are a striking example of phenotypic diversity, yet the sources of selection that allow different morphs to persist within populations remain poorly understood. In particular, despite the importance of aggression in mediating social dominance, few studies have considered how heterospecific aggression might contribute to the maintenance or divergence of different colour morphs. To redress this gap, we carried out a field-based study in a Nicaraguan crater lake to investigate patterns of heterospecific aggression directed by the cichlid fish, *Hypsophrys nicaraguensis*, towards colour polymorphic cichlids in the genus *Amphilophus*. We found that *H. nicaraguensis* was the most frequent territorial neighbour of the colour polymorphic *A. sagittae*. Furthermore, when manipulating territorial intrusions using models, *H. nicaraguensis* were more aggressive towards the gold than dark colour morph of the sympatric *Amphilophus* species, including *A. sagittae*. Such a pattern of heterospecific aggression should be costly to the gold colour morph, potentially accounting for its lower than expected frequency and, more generally, highlighting the importance of considering heterospecific aggression in the context of morph frequencies and coexistence in the wild.

## 1. Introduction

A major endeavour of evolutionary ecology is to understand the processes that underlie the remarkable diversity that exists both within and between species. One of the most striking examples of this diversity is seen in polymorphic species that exhibit consistent genetic variation in coloration within populations [1–3]. Indeed, studies of such species have provided evolutionary biologists with important insights into mechanisms underpinning individual variation and phenotypic diversity, as well as mechanisms of speciation [2–6]. Yet, despite these insights, the actual sources of selection that allow the persistence of different morphs within the same population remain poorly understood [3].

In the absence of specific selection pressures that permit the coexistence of different morphs, a better performing morph would soon be expected to drive others from the population. Even in the absence of any performance advantages, one might expect the type of variation represented by discrete morphs to simply erode from the population through genetic drift and other random events [7,8]. However, morph coexistence can be favoured if a rarer morph has an advantage that is tempered at higher frequencies [9–13].

One mechanism that can act as a powerful selective force affecting the evolution of colour polymorphisms and morph frequencies is aggressive interactions. For example, individuals of a particular colour morph may enjoy fitness advantages from being more aggressive than others [14,15]. In Gouldian finches (*Erythrura gouldiae*), for instance, red-headed birds, which naturally occur at lower frequencies in the wild, aggressively dominate the more common black-headed individuals. This advantage, however, is counterbalanced at higher frequencies by stress costs of frequent aggressive interactions [16,17]. Yet, despite the apparent importance of aggression, few studies have considered its role in contributing to the maintenance or divergence of different phenotypes.

Among the few notable exceptions, the focus has almost exclusively been on patterns of aggression between the different phenotypes or morphs [18,19]. However, it is important to note that competitive biases may have evolutionary consequences not only within species, but also in the context of heterospecific interactions. For example, in sympatric species of *Hetaerina* damselflies, heterospecific intruders elicited less aggression than conspecific intruders in species pairs with dissimilar wing coloration. By contrast, in species pairs where wing coloration was more similar, heterospecific intruders were attacked as aggressively as conspecifics [20]. Such biased aggressive encounters can have important ecological and evolutionary consequences by affecting patterns of species distribution and coexistence [21]. Indeed, aggressive interactions between species are actually very common, and can be as intense as aggression within a species [22,23]. Nevertheless, the evolutionary consequences of heterospecific aggression have often been overlooked [22–24].

The crater lakes of Central America provide an excellent experimental setting for investigating the role of heterospecific aggression on patterns of coexistence and relative colour morph frequencies. Among the species of cichlid fish that coexist in these lakes, the most prominent are members of the Midas cichlid complex *Amphilophus* spp. (*sensu lato* [25–27]). Many species within this group display discrete, but naturally co-occurring, and genetically inherited ‘dark’ and so called ‘gold’ (typically orange in colour) phenotypes, i.e. ‘morphs’ [26,28]. In the wild, territory owners bias their aggression towards opponents that are of the same colour morph as themselves [19]. Given that different colour morphs share the same habitats, such a pattern of aggression is expected to benefit whichever colour morph has the lowest frequency, helping to explain how a novel morph can establish in a population and then coexist with other morphs [18,21]. Furthermore, in the laboratory, gold individuals socially dominate similar-sized, dark coloured fish [28]. Nevertheless, in polymorphic populations, typically only 10% or less of the adult individuals are of the gold morph, with the rest being dark [26,29]. Thus, additional selection pressures are probably at play in countering the frequency-dependent advantage of the gold morph. In this regard, it has been suggested that a higher predation risk may select against gold individuals [28,30,31] but the evidence has so far been mixed [32–34] and, hence, it is unlikely that differences in predation (if any) are solely responsible for the low frequency of gold morph individuals in natural populations.

In the current field-based study, we considered another possibility: interspecific aggression. Specifically, we experimentally investigated territorial aggression, as displayed by a key heterospecific territorial competitor, towards dark and gold individuals in colour polymorphic *Amphilophus* cichlid fish. In particular, we assessed the possibility that heterospecific aggression is biased in a way that has the potential to contribute to colour morph frequencies encountered in the wild.

## 2. Material and methods

### (a) The study system

This field-based study was conducted in Lake Xiloá, Nicaragua (latitude 12° 12.8' N; longitude 86° 19.0' W) over the course of two breeding seasons (December 2010–January 2011 and December 2013–January 2014). The lake supports more than 10 cichlid species



**Figure 1.** Attacking male Nicaragua cichlid, *Hypsophrys nicaraguensis*, Lake Xiloá, Nicaragua.

whose breeding seasons are largely overlapping [35–37]. Typically, irrespective of species, when pairs are ready to spawn, they claim a territory on the lake floor, and then aggressively defend the territory until the juveniles are ready to disperse [35,38]. This aggressive behaviour is directed against both conspecific and heterospecific individuals [35,37], with the patterns of niche overlap and commensal interactions potentially fine-tuning the intensity of aggression [35,38,39]. Thus, territorial aggression is directed towards competitors for territory space (both conspecific and heterospecific), brood predators (both conspecific and heterospecific), as well as conspecific sexual competitors [35,37,40].

Here, we focused on patterns of heterospecific aggression directed towards colour polymorphic *Amphilophus* species that share their breeding grounds with other, concurrently breeding cichlid species. One such species is the Nicaragua cichlid, *Hypsophrys nicaraguensis* (also known as the butterfly cichlid, parrot cichlid and moga; figure 1). Anecdotal evidence suggests that *H. nicaraguensis* parents are very effective at excluding many species from close proximity to their territories [38]. Previous work also suggests that *H. nicaraguensis* breeding pairs are often neighbours with *Amphilophus sagittae* and, to a lesser extent, *Amphilophus xiloensis* ([19,35], T.K.L. 2010–2014, personal observations). Both of these *Amphilophus* species exhibit the distinct gold and dark colour morphs, with the frequency of the gold morph being below 10% and close to 20% in *A. sagittae* and *A. xiloensis*, respectively [29].

Focusing on the interactions between *H. nicaraguensis* and the colour polymorphic *Amphilophus* species with which it co-occurs, our study comprised two distinct components. First, we carried out underwater field surveys to quantify the territorial neighbours of breeding pairs of both *H. nicaraguensis* and *A. sagittae*, the former being putatively the most common colour polymorphic neighbour of the latter (see the electronic supplementary material for detailed field survey methods). The aim here was to confirm that these taxa do, indeed, share overlapping territorial distributions. Second, we carried out a field experiment to investigate the aggressive responses of *H. nicaraguensis* when presented with dummy models of sympatric cichlid species, including models of gold and dark coloured *A. sagittae* and *A. xiloensis*. This was done to test whether, in fact, heterospecific aggression is biased towards individuals of a particular colour morph—a pattern of aggression that could help to elucidate the observed colour morph frequencies in the wild. The methods of this experiment are detailed below.

### (b) Aggression biases by *Hypsophrys nicaraguensis*

We experimentally assessed heterospecific aggression by the focal territorial species, the Nicaragua cichlid, *H. nicaraguensis*.

A scuba diver sequentially presented *H. nicaraguensis* pairs with four different stimulus types (with a haphazard order of presentation for each *H. nicaraguensis* pair; see below): (i) *Amphilophus* (*A. sagittae* and *A. xiloensis*) individuals of the dark morph in breeding coloration (mostly solid dark); (ii) *Amphilophus* individuals of the dark morph in non-breeding coloration (grey with dark vertical markings); (iii) *Amphilophus* individuals of the gold morph (which, in contrast with the dark morph, looks the same irrespective of whether or not it is breeding) and (iv) individuals of the jaguar cichlid, *Parachromis managuensis*, from Lake Xiloá in adult coloration. The latter is a species whose background coloration can range from yellowish to beige, and was chosen as a control to allow us to disentangle between a more general aggressive response towards light-coloured intruders and a specific aggression toward the lighter (i.e. gold) morph of the *Amphilophus* species.

We controlled for the stimulus phenotype and behaviour by using model intruders that were all the same size (16 cm in length). Hand-made models have been successfully used as stimuli to elicit ecologically relevant behaviours in a range of fish species (reviewed in [41]), including field-based studies of territorial aggression in cichlids [19,42,43]. The use of models (or 'dummies') have a significant advantage over the use of live stimulus animals, by allowing us to explicitly control for confounding factors that might otherwise arise from differences in the behaviour of the stimulus animals. However, instead of using the more traditional wax or painted shapes employed by other researchers [41,44], we chose to use more realistic-looking models based on photographs of wild-caught fish following the methods of Lehtonen [19]. Specifically, the intruder models were made by gluing waterproof photographic colour prints of a photo of a lateral side of a live or freshly euthanized fish of the desired type onto both lateral sides of a fish-shaped floating plate (thickness = 6 mm). The model was then attached to a sinker with a thin, transparent fishing line, allowing it to float in a natural position approximately 15 cm above the lake bottom. The numbers of models prepared this way were as follows for the four types of stimuli: (1) *Amphilophus* dark in breeding coloration:  $n = 11$ , (2) *Amphilophus* dark, non-breeding coloration:  $n = 11$ , (3) *Amphilophus* gold (looks the same independent of the breeding phase):  $n = 12$ , and (4) *P. managuensis*:  $n = 10$ . For each of the stimulus types involving *Amphilophus* (types 1–3), we had photographed both of the colour polymorphic Lake Xiloá species, *A. sagittae* and *A. xiloensis*. In half of the replicates, we used *A. sagittae* models exclusively (for the stimuli types 1–3), and *A. xiloensis* models were used for the rest of the replicates. Including both *A. sagittae* and *A. xiloensis* models provided us with the opportunity to test whether any differences in aggression directed by *H. nicaraguensis* towards gold and dark morphs is species specific or a more generalized response towards the different colour morphs. The *P. managuensis* models (stimulus type 4) were prepared using photographs of adult specimens from Lake Xiloá. Each model ( $n = 44$ ) was prepared using a photograph from a different fish. A unique combination of the four different model types was used in each of our 24 replicates (see below).

Each replicate was initiated by placing a dummy at a distance approximately 40 cm from the centre of the *H. nicaraguensis* territory. We then counted the total number of aggressive encounters by territory-owners (both male and female) towards the dummy for 5 min, giving the total 'aggression rate' [19]. After a 5 min resting period (with all models out of sight), we repeated the procedure with each of the remaining model types, one after the other, so that each pair of territorial *H. nicaraguensis* was exposed sequentially to all four types of models. The 5-min resting period was chosen for two main reasons. First, we wanted to minimize any temporal changes in both abiotic (e.g. lighting) and biotic (e.g. other fish moving in and out of the area) conditions. Second, we considered a 5-min interval to be ecologically relevant as territory holders commonly encounter a range of different

species within a span of a few minutes ([37], personal observations). We sampled 24 biparentally defended *H. nicaraguensis* territories, with every possible order of presenting the four different model types ( $n = 24$  different combinations) used only once to control for any potential order effects.

To assess the effects of the type of the model intruder (1–4), the species of *Amphilophus* used for the models (*A. sagittae* versus *A. xiloensis*), the sex of the focal territory holders (male versus female) and interactions between these effects, we analysed the aggression data using a generalized mixed model with a negative binomial error distribution, as appropriate for over-dispersed count data [45]. To account for non-independence of the actions of a male and female defending a territory, territory ID was added as a random effect. We simplified the model in a stepwise fashion by assessing whether we could refit the model without the least significant term of the highest remaining order. We applied  $\chi^2$ -tests (with  $p = 0.05$  as the cut-off point) for this purpose. We used R v. 3.1.0 software (R Development Core Team) for the analyses.

### 3. Results

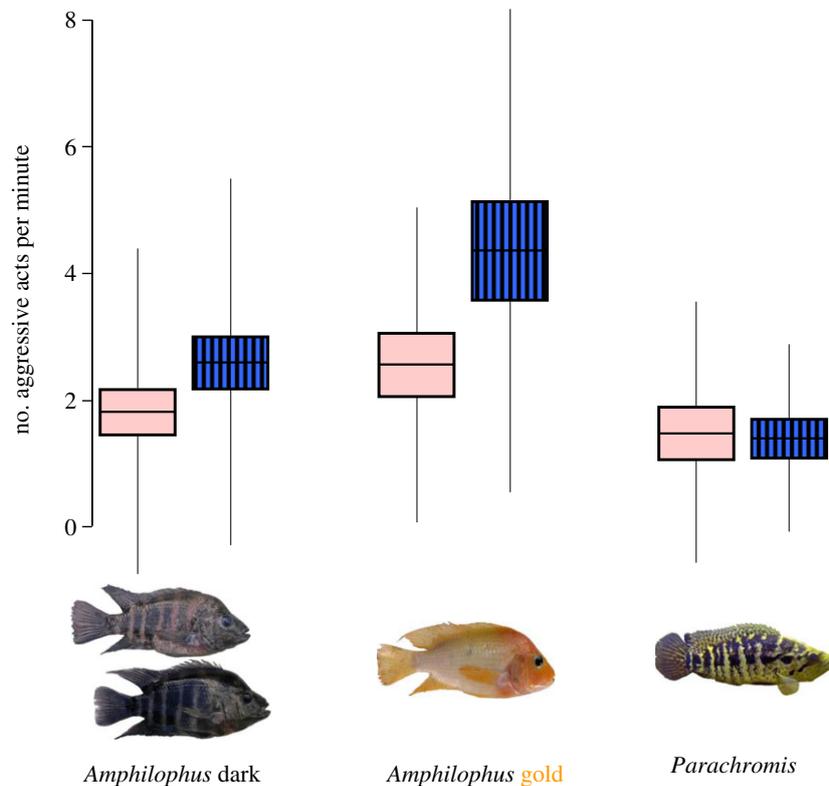
#### (a) Field survey: distribution of territorial neighbours

For the focal *H. nicaraguensis* territories ( $n = 113$ ), the most common nearest neighbours were conspecifics (34%, or  $n = 38$  territories). In turn, 25% of the territories ( $n = 28$ ) had a pair of *A. sagittae* as the closest neighbour, of which 26, 0 and 2 were dark  $\times$  dark, gold  $\times$  gold and mixed colour pairs, respectively (electronic supplementary material, table S1).

Focusing on *A. sagittae* territories ( $n = 200$ ), the most common neighbours were *H. nicaraguensis* ( $n = 104$  territories, or 52%), followed by conspecifics ( $n = 43$ ; 21.5%). Regarding the colour morphs of these focal territory holders, the proportion of *H. nicaraguensis* neighbours was 44.5% (45/101), 50% (21/42) and 66.5% (38/57) for dark  $\times$  dark, gold  $\times$  gold and mixed pairs, respectively (electronic supplementary material, table S1). This indicates no significant difference between the different *A. sagittae* pair types in how often they had *H. nicaraguensis* as the closest neighbour (G-test of independence with Williams' correction,  $G = 2.142$ , d.f. = 2,  $p = 0.34$ ). Hence, in line with our prediction, *H. nicaraguensis* was a very frequent territorial neighbour of both colour morphs of *A. sagittae*. See the electronic supplementary material, table S1 for the complete list by species of the closest territorial neighbours.

#### (b) Aggression biases by *Hypsophrys nicaraguensis*

When we applied a generalized mixed model to assess the rate of aggression (in relation to the territory-holder sex, intruder model type and whether *Amphilophus* intruder models were *A. sagittae* or *A. xiloensis*), we found all interactions to be non-significant (in all cases  $p > 0.10$ ). Similarly, there was no significant difference in aggressive responses of *H. nicaraguensis* territory holders towards *A. sagittae* and *A. xiloensis* intruder models ( $\chi^2 = 0.01$ , d.f. = 1,  $p = 0.92$ ). A model refitted with the remaining effects, i.e. territory-holder sex and type of the model intruder, indicated that there was no significant difference in aggression directed to the breeder versus non-breeder models of the dark morph ( $z = 1.16$ ,  $p = 0.25$ ). We, therefore, fitted a new model in which the two were combined ( $\chi^2 = 1.34$ , d.f. = 1,  $p = 0.25$ ). This final model showed that males were more aggressive than females ( $z = 3.80$ ,  $p = 0.0001$ ; figure 2)



**Figure 2.** The total rate of aggression towards model opponents by female (pink boxes) and male (blue boxes with vertical stripes) *Hypsophrys nicaraguensis*. The different types of model intruders are pictured at the bottom of the graph. Central horizontal lines within the boxes, margins of the boxes and whiskers indicate means, s.e. and s.d., respectively. The number of *H. nicaraguensis* territories sampled, and hence the sample size for each box, was 24.

and aggression was significantly higher towards the gold than dark morph models ( $z = 3.03, p = 0.0024$ ; figure 2). In turn, compared with the control (*Parachromis*) models, aggression rate towards dark morph models (breeders and non-breeders combined) tended to be higher (figure 2) but not significantly so ( $z = 1.80, p = 0.072$ ).

#### 4. Discussion

We found that *H. nicaraguensis* is a key territorial neighbour of *A. sagittae* in Lake Xiloá. This result suggests a high potential for territorial interactions between the two species, which is supported by our underwater observations during this and earlier studies [19,38]. Indeed, as competition within and between species for territory space can be intense in multi-species breeding grounds, such as those occupied by many *Amphilophus* species [35,46,47], a high occurrence of these interspecific disputes is likely to have a significant effect on the territory-holders' success in territorial acquisition and maintenance. In this respect, while interactions with conspecific competitors probably have the greatest impact on territory acquisition in *A. sagittae*, our results suggest that *H. nicaraguensis* is the most important of the heterospecific competitors. In particular, approximately half of *A. sagittae* territories, independent of the colour morph of the territory holders, had a pair of *H. nicaraguensis* as their closest neighbour. Interestingly, close proximity of a *H. nicaraguensis* pair has been suggested to benefit small cichlids, possibly due to *H. nicaraguensis* being aggressive towards many larger species [38]. In this respect, the high level of aggression that *H. nicaraguensis* directs towards the gold-coloured fish (see below) may contribute to the complete absence of gold  $\times$  gold *A. sagittae* pairs as the nearest neighbours of

the focal *H. nicaraguensis* territory holders. However, this pattern of neighbouring territories could similarly be explained by the low frequency of the *A. sagittae* gold morph [29], especially given that when we focused on *A. sagittae* territories, *H. nicaraguensis* was found to be the most common neighbouring species independent of the colour morph(s) of the territory holders. Because of the deeper water depth preferred by breeding *A. xiloensis* and their lower abundance at the study site ([29], personal observations), neither colour morph of this species was among the nearest neighbours in the current assessment of territorial interactions. Our observations nevertheless suggest that at the sites where *H. nicaraguensis* and *A. xiloensis* are neighbours, they may similarly compete for territory space and potentially other resources. Indeed, the results of our model presentation experiment indicated that *H. nicaraguensis* reacted similarly towards *A. sagittae* and *A. xiloensis* models. The results also remain qualitatively the same even if *A. xiloensis* models are excluded.

In our intruder model presentation experiment, *H. nicaraguensis* directed more aggression towards gold than dark morph *Amphilophus* models, regardless of whether the latter were in breeding or non-breeding coloration. This result suggests that gold-coloured *A. sagittae* trying to establish a territory are likely to experience more aggression than dark *A. sagittae* by their key heterospecific competitors, *H. nicaraguensis*. Being subject to a higher rate of aggression should be costly to the gold morph. For example, the proportion of gold individuals—relative to dark ones—that are able to establish, and successfully maintain, an adequate territory may be lower than it would be in the absence of the biased heterospecific aggression. Interestingly, such a disadvantage could help to explain the morph distributions observed in the wild, e.g. the lower than expected frequency

of individuals of the gold compared with dark morph in *A. sagittae* [19,29]. Furthermore, the different breeding habitat preferences of *A. xiloensis* [29] may allow that species to have less contact with many other cichlid species, such as *H. nicaraguensis*, potentially explaining why it has a higher frequency of gold individuals than *A. sagittae* [29]. Indeed, if such biases in heterospecific aggression are also displayed by other species besides *H. nicaraguensis*, heterospecific aggression could help to explain the low frequencies of the gold morph more generally, especially within the Midas cichlid complex. Our results, therefore, suggest that heterospecific aggression should be taken into account when the complex interactions that are involved in maintenance of a stable (colour) polymorphism [2–4,48] are considered.

Why should *H. nicaraguensis* territory holders be more aggressive towards gold than dark *A. sagittae*? One possible explanation is that the gold morph individuals of *A. sagittae* appear more similar in colour to *H. nicaraguensis* than dark individuals (figures 1 and 2). In particular, phenotypically more similar species and individuals are known to be treated more aggressively than species (or individuals) that are less alike with the aggressor [20,49–52], as has been shown, for example, in the conspecific context, in Lake Xiloá for *A. sagittae* [19]. Such a pattern of aggression is usually assumed to stem from territory holders recognizing intruders similar to themselves as more serious competitors [18,24]. Currently, we do not have data for assessing whether the two colour morphs differ in terms of their status as competitors or threat to *H. nicaraguensis*. Such a threat difference is, however, feasible because motivational states of dark individuals may be more evident than those of gold individuals, because of the latter having a lower capacity to signal through adjustment of colour patterns [28,31]. In this respect, one prediction of the ‘uncertainty hypothesis’ (*sensu* Peiman & Robinson [22]) is that individuals should be more wary towards intruders whose intentions are uncertain. According to this hypothesis, *H. nicaraguensis* should be particularly alert when interacting with the gold morph.

It is also possible that the bright coloration of the gold morph *per se* might have resulted in a higher level of aggression directed towards them, with orange and reddish coloration being often associated with aggressive signalling and interactions in a wide range of taxa. In particular, not only are individuals and morphs with such colours commonly dominant in interactions among conspecific individuals, as in the Gouldian finch ([53] and references therein), but brightly coloured individuals may also be challenged more aggressively, as has been shown in reptiles [54] and fish [55,56], including cichlids [57]. Indeed, although niche overlap has been offered as one of the main predictors of the level of heterospecific aggression [22], it does not always explain its intensity, as in lacertid lizards [58]. In cichlids, it also remains possible that because the dark individuals are—and historically have been—much more numerous [26,29,59], *H. nicaraguensis* territory holders may be more familiar with this intruder type and, because of learning effects such as the ‘dear enemy effect’ and stimulus habituation [60,61], react more intensively to less familiar gold individuals. Furthermore, because of the considerably lower background matching ability of the gold morph [31], individuals of the gold morph are likely to be more conspicuous to *H. nicaraguensis* territory holders.

The possibility that *H. nicaraguensis* territory holders were simply reacting to phenotypic similarity between

themselves and the intruders might predict that they should also be more aggressive towards the *P. managuensis* models that—similarly to them—have a paler (yellowish and sometimes beige) coloration (figure 2). This, however, was not the case: if anything, *H. nicaraguensis* territory holders directed less aggression toward *Parachromis* than any other model type. One explanation for low rates of aggression between the species is that they seem to venture into each other’s territories much less often than *H. nicaraguensis* and *A. sagittae* do (personal observations): in the current study none of the assessed *H. nicaraguensis* territories had *Parachromis* territories as their close neighbours. More generally, compared with the *Amphilophus* species, the potentially smaller niche overlap between *Parachromis* and *H. nicaraguensis* could explain why *H. nicaraguensis* might direct a reduced level of aggression towards the former; heterospecific aggression is generally predicted to have a positive association with the magnitude of niche overlap [22]. We also note that although small *Parachromis* in their typical dark juvenile coloration often predate upon fry of other cichlids ([37], personal observations), we have never observed larger *Parachromis* individuals in the paler adult coloration to do so, despite having conducted extensive fieldwork in the area (e.g. [19,29,38,47]), suggesting that they might not pose a direct threat to cichlid parents. This could be relevant as all of the *P. managuensis* models used in our study displayed adult coloration. Intriguingly, *H. nicaraguensis* sometimes engage in a peculiar species interaction with *Parachromis dovii*, in which a male of the former appears to help the latter in territory defence [39]. We cannot exclude the possibility that this species interaction could also contribute to the lower observed rates of aggression between *H. nicaraguensis* and *P. managuensis*, with the latter being quite similar in appearance to *P. dovii* (personal observations).

To conclude, the results of this study show that *H. nicaraguensis* is capable of a sophisticated assessment of territorial intruders based on their phenotype alone when controlling for any behavioural differences (by using model intruders). In particular, they were more aggressive towards the gold than dark morph of *A. sagittae* and *A. xiloensis*, while tending to show less aggression towards yellowish/pale *Parachromis* models. Importantly, the higher rate of aggression directed towards gold *Amphilophus* individuals is likely to be an important cost that could help to explain their lower than expected frequency. More broadly, greater attention should be given to the role of heterospecific aggression in explaining observed patterns of morph frequencies and coexistence.

**Ethics.** MARENA (Nicaragua) approved this non-intrusive field-based study and issued the permit for it (permit no. 013-102013).

**Data accessibility.** Our data files have been uploaded to Dryad: <http://dx.doi.org/10.5061/dryad.18v6q>.

**Authors’ contributions.** T.K.L. conceived the study, performed the experiment, analysed the data, wrote the first draft of the manuscript and together with B.B.M.W., designed the study. All authors participated in preparations for the experiment and writing the manuscript, and approved the final version of it.

**Competing interests.** We declare we have no competing interests.

**Funding.** The study was funded by University of Turku (to T.K.L.), Monash University (to B.B.M.W.) and the Holsworth Wildlife Endowment Fund and the Linnean Society of New South Wales (both to W.S.).

**Acknowledgements.** We thank Christopher Anderson, Marta Barluenga, Eric van den Berghe, Karine Gagnon, Lasse & Pirjo Lehtonen, Ken McKaye, Mark McKaye and an anonymous reviewer.

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RESEARCH ARTICLE

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# Allopatry, competitor recognition and heterospecific aggression in crater lake cichlids

Topi K. Lehtonen<sup>1,2\*</sup>, Karine Gagnon<sup>1</sup>, Will Sowersby<sup>2</sup> and Bob B. M. Wong<sup>2</sup>

## Abstract

**Background:** Aggressive behaviour can have significant evolutionary consequences—not only within species, but also in the context of heterospecific interactions. Here, we carried out an experimental field study to investigate the importance of phenotypic similarity on levels of aggression between species whilst controlling for familiarity effects using manipulated allopatric stimuli. Specifically, we investigated aggressive responses of territory holding males and females in two species of Neotropical cichlid fish, *Amphilophus sagittae* and *Hypsophrys nicaraguensis*, that differ in their phenotypic similarity to our allopatric stimulus species, *Amphilophus astorquii*.

**Results:** We found that, independent of phenotypic similarity (and correlated phylogenetic proximity) between the territory holders and intruder, territorial aggression was not adjusted in relation to allopatric intruder colour markings that are associated with different levels of threat and known to provoke different responses in a sympatric setting. We also found that males and females did not differ in their overall patterns of aggression adjustment towards intruder cues. Nevertheless, the two focal species, which share the same breeding grounds and external threats, exhibited different sex roles in breeding territory defence.

**Conclusion:** Together with earlier studies assessing heterospecific aggression in sympatry, our current results highlight the importance of coevolution and learning in species interactions.

**Keywords:** Allopatry, Behavioural plasticity, Cichlid fish, Colour signal, Competitor recognition, Heterospecific aggression, Phenotypic similarity, Signal reliability, Species interaction

## Background

Heterospecific aggression tends to be more pronounced among congeneric, phenotypically similar species as compared to aggression directed to those from other genera and different phenotypes [1, 2]. Besides the extent of niche overlap, competitor recognition is assumed to play a significant role in the evolution of heterospecific aggression [1, 2]. Indeed, to properly adjust its aggressive responses, a territory holder needs to be able to correctly recognize (heterospecific) intruders that pose different levels of threat [3, 4]. Here, individuals may rely on similar sensory and cognitive means for recognising phenotypically similar heterospecifics as they would for conspecifics [5]. As a consequence, it may be easier for

territory holders to appropriately adjust aggression towards those heterospecifics to which they have a higher phenotypic resemblance. Likewise, a novel heterospecific signal may be easier to detect when it is similar to a familiar signal [6].

Signal recognition can be driven not only by niche overlap or phenotypic resemblance between interacting species [2, 6, 7], but also by learning opportunities. For example, blue-coloured males in *Pundamilia* cichlid fish adjust their aggression depending on their prior exposure to red (as opposed to only blue) males [8]. Such effects can also be sex-specific, as shown in female damselflies, with species recognition in a mating context being affected by their prior experience with conspecific and heterospecific males [9]. The result of a recent meta-analysis also suggests that species recognition, at least in a mating context, may have evolved quite differently between the sexes, with the capacity to discriminate between

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conspecifics and heterospecifics being based more on learning in females [5]. Consistent with such an interpretation, the sexes are often subject to divergent selection pressures in terms of, for example, aggressive behaviour and parental roles [10, 11] while, at the proximate level, they may also differ in cognitive abilities in colour or pattern recognition [12, 13]. Based on such findings, it is conceivable that the sexes could also differ both in their opportunity and ability to recognise territorial intruders, a prediction that has hitherto been subject to very little empirical attention [5].

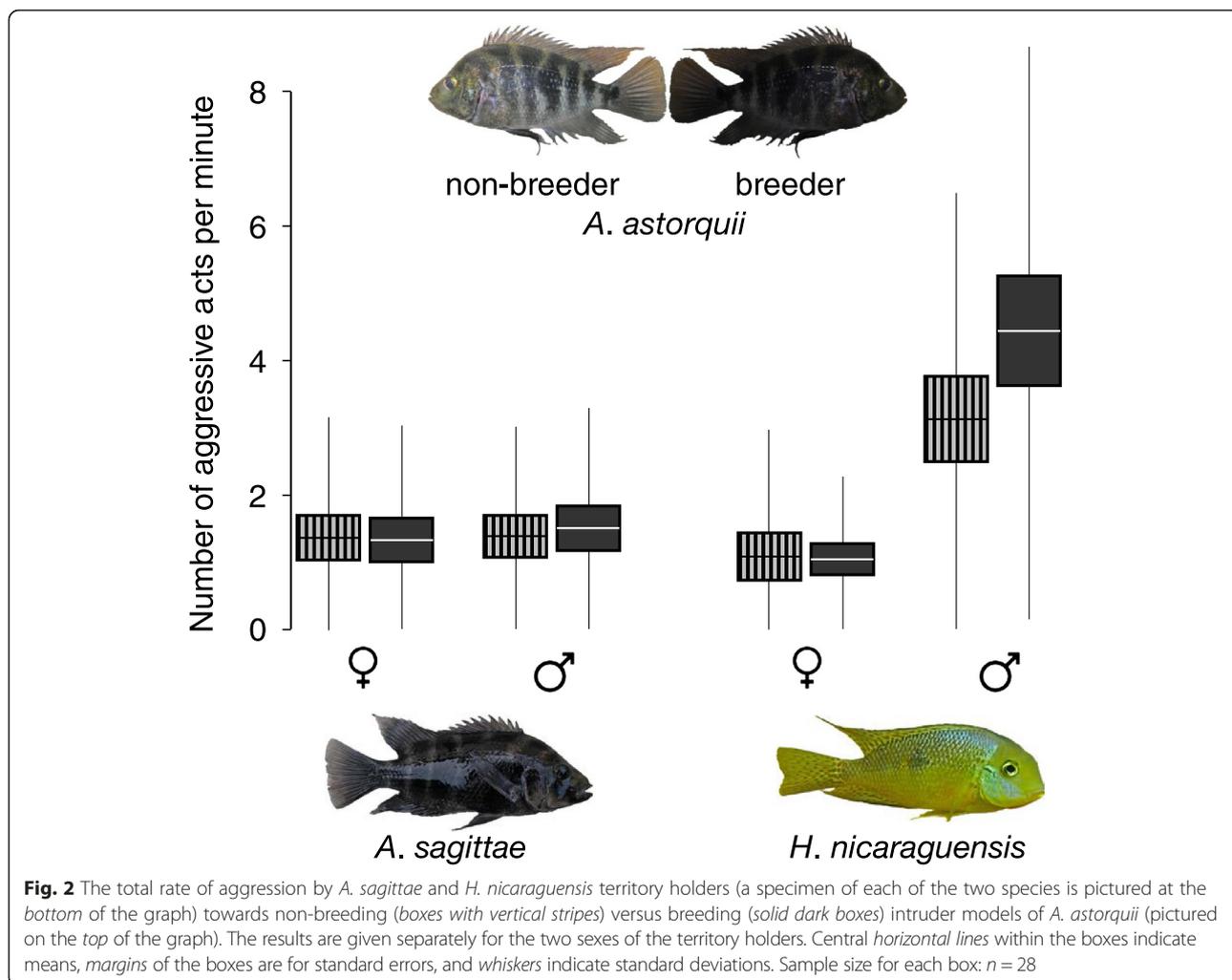
In fish, colour cues have often evolved to play an important role in species recognition, in both a competition and reproduction context. This is especially true in cichlids [14, 15]. For instance, in the Central American Midas cichlid species complex (within the genus *Amphilophus*, see [16, 17]), experiments with manipulated (i.e. 'dummy') stimuli have found that coloration alone is a sufficient cue for competitor recognition both within [18] and among sympatric species [19]. Earlier results on African cichlids also indicate that phenotypic similarity may affect heterospecific aggression at the species (or morph) level [20, 21]. However, we currently know far less about whether individuals, when reacting to another species, are capable of adjusting their aggression according to differences in threat levels posed by different individuals of that species [22], or how familiarity or opportunities for learning may affect the adjustment of such aggression [5]. Accordingly, in a field-based experiment, we tested the influence of phenotypic similarity on aggression, as directed by breeding territory holders towards heterospecific 'intruders' in cichlids living in Nicaraguan Crater Lake Xiloá. Our study focused on two species of territory holders, *Amphilophus sagittae* and *Hypsophrys nicaraguensis*, which differed in their phenotypic similarity to the allopatric intruder species, *Amphilophus astorquii*, with which they were presented. The intruder species used in our study is allopatric with the two focal species, allowing us to control for any behavioural differences that might arise due to prior experience with the stimulus.

When ready to spawn, pairs of these cichlid fish species claim a sedentary breeding territory, which they aggressively defend for approximately a month after their fry have become free-swimming [23–26]. This aggression is directed towards both competitors (especially for territory space) and brood predators that can be conspecific, congeneric, as well as more distantly related species [25, 27]. In this respect, not all intruders pose the same level of threat. For instance, breeding individuals are likely to represent a lower threat than non-breeders, as the former have already claimed, and are busy defending, a territory and offspring of their own (and relying on previously accumulated energy reserves to do so), instead of actively seeking prey. In contrast, non-breeding individuals are much

more likely to attempt to prey upon eggs and juveniles of both conspecifics and heterospecifics [22, 25, 26, 28]. Supporting this scenario, an earlier observational study of fish in Crater Lake Apoyo (Nicaragua) showed that non-breeding *A. astorquii* are subjected to more intense aggression than breeders, by both conspecific and congeneric (*Amphilophus zalius*) territory holders [22]. Importantly, breeding and non-breeding individuals (both males and females) of *A. astorquii*—as well as those of *A. sagittae* and *A. xiloaensis* in Crater Lake Xiloá—have strikingly different body markings: in contrast to the uniformly dark colour of the breeders, non-breeders have contrasting dark and light vertical bars along their flanks ([22]; Figs. 1 and 2). In addition, another recent study shows that for *A. sagittae* territory holders, colour patterning alone is a sufficient cue for directing more aggression towards model intruders with non-breeder colour markings than with breeder coloration, when these are look-alikes of the sympatric and congeneric species *A. xiloaensis* [19]. In contrast to the above-mentioned *Amphilophus* species, our other focal territorial species, *H. nicaraguensis*, does not exhibit any clear differences in body markings between breeding and non-breeding phases. It does, however, share the breeding habitat with multiple *Amphilophus* species (including *A. sagittae*), and is likely to be subject to similar ecological pressures. In this shared environment, *H. nicaraguensis* has to compete for territory space with *Amphilophus* species, and also defend its juveniles against them ([25], personal observations). Compared to the two *Amphilophus* species, *H. nicaraguensis* also has much more pronounced sexual size dimorphism, with males of *H. nicaraguensis* often reaching the size of small adult *A. sagittae* ( $\geq 15$  cm total length), while female *H. nicaraguensis* are considerably smaller (typically below 10 cm) [25, 29]. Male and female *H. nicaraguensis* also have slightly different colour markings, with the latter possessing a prominent dark lateral stripe. By contrast, male and female *Amphilophus* do not differ in coloration, although males within a



**Fig. 1** *Amphilophus sagittae* territory holders attacking a model of *A. astorquii* with non-breeding body markings. The male is closer to the camera, with the female only partially visible behind him



pair are slightly larger [25, 27, 29] and tend to have longer fin filaments than females.

Breeding and non-breeding individuals posing different threat levels to territory holders allowed us to test whether territory holders adjust their aggression depending on the presumed threat status of heterospecific intruders. We hypothesised that body colour patterns related to differences in breeding status in *A. astorquii* intruders would more likely influence territorial aggression in the congeneric and phenotypically similar *A. sagittae* compared to the dissimilar *H. nicaraguensis* (Fig. 2). Specifically, if recognition of intruder breeding status (based on coloration) is stronger when the intruder is phenotypically similar (see [2]), we would expect *A. sagittae* territory holders to make a clearer distinction between *A. astorquii* intruders differing in breeding status than *H. nicaraguensis* territory holders. The use of an allopatric intruder, *A. astorquii* (which is endemic to Lake Apoyo), controlled for the opportunity for stimulus learning. This is relevant because familiarity is known to often affect aggression in general

[30–32], with opportunities for learning potentially also influencing heterospecific aggression [8, 9]. Similarly, if the sexes differ in their sensitivity towards the type of intruder (e.g. due to different abilities in heterospecific recognition, sensu Ord et al. [5]), we would also expect to see the pattern of responses to the different intruder types to be sex-specific (i.e. we would expect to find evidence of a sex  $\times$  intruder type interaction).

## Methods

This field-based study was conducted using SCUBA in Lake Xiloá, Nicaragua (12°12.8' N; 86°19.0' W) between December 2013 and January 2014, during the breeding season of our two focal species, *Amphilophus sagittae* and *Hypsophrys nicaraguensis* ([23, 24], personal observations). In particular, we investigated the effect of phenotypic similarity on the level of territorial aggression towards non-breeders vs. breeders, while controlling for the opportunity for stimulus learning by using an allopatric intruder, *A. astorquii*. In addition,

we explicitly controlled for any effects that might otherwise be caused by the behaviour of the stimulus, by using intruder models (or ‘dummies’). Such models have been successfully used to study behaviour in a range of fish species (reviewed by Rowland [33]), including *Amphilophus* [18, 19, 34] and other cichlids [35–37]. Instead of the stylised fish models that have been used in past studies [33–35], we opted for more realistic-looking models based on photographs of wild-caught fish following the methods of Lehtonen [18]. Specifically, we glued a waterproof, photographic colour print of the lateral side of a live or freshly euthanized specimen (sex unknown or not noted) onto each lateral side of an elliptical floating plate with a thickness of 6 mm. All our models were 16 cm long and attached to a sinker with a thin, transparent fishing line, so that they floated in a natural position approximately 15 cm above the lake bottom during the trials ([18, 19, 38]; Fig. 1). Models of this size were easy to handle under water and represented an overlap in the size ranges of adult male and female *A. sagittae* [19] as well as male *H. nicaraguensis*.

In our model presentation (see below), the breeder and non-breeder models (based on 24 different *A. astorquii* individuals) were paired. In 12 model pairs, the non-breeder model was, as explained above, based on a photo of a non-breeding individual, which was then also used to generate a ‘breeding’ counterpart by manipulating the image in Adobe Photoshop (Adobe Systems, Inc., San Jose, CA, USA) until it resembled a fish with the uniform (i.e. non-barred) markings of a breeding individual. Each ‘breeder’ counterpart had therefore exactly the same shape and posture as compared to its ‘non-breeder’ model pair, with colour markings being the only difference between the two models. For the rest ( $n = 12$ ), in turn, the non-breeder counterpart model was made by manipulating the image of a breeding individual (in terms of shading and contrast) so that the horizontal bars became visible and the fish resembled a non-breeder (Figs. 1 and 2). Hence, in total we had 24 fixed model pairs, with each model pair made using a photograph from a different individual. These 24 models were presented to 28 *A. sagittae* and 28 *H. nicaraguensis* territory-holding pairs defending small fry in a habitat characterised by pebbles lying on a finer substratum of sand and organic material. As a result, four model pairs were used twice for both focal species, while the rest of the models were used only once per species. This design was accounted for in the statistical analyses (see below).

Each trial started by placing an *A. astorquii* model approximately 40 cm from the centre of the focal territory, which is at, or slightly below, an average distance territory holders swim when deterring territorial intruders [22, 27, 39]. We counted the total number of aggressive responses (either slow movement toward the model with flared gills and fins in a threat display or a rapid advance

sometimes followed by a bite [18, 19]) by both male and female territory owners towards the model for 5 min (giving the total aggression rate sensu [19, 27]). We then removed the model from sight for a predetermined rest period of 5 min, after which we repeated the above with the alternative model (i.e. a breeder model if the initial model was a non-breeder and vice versa). The model type (breeder versus non-breeder) presented first was randomised. After the trial, the territory was marked with a numbered piece of ceramic tile to avoid assessing the same territory more than once.

### Statistical analyses

To assess the effects of the species (*A. sagittae* versus *H. nicaraguensis*) and sex (male versus female) of the focal territory holder, as well as the type of model intruder (breeder versus non-breeder), we applied a generalized mixed model using the ‘glmmPQL’ function of the packages ‘nlme’ and ‘MASS’ with a negative binomial error distribution appropriate for over-dispersed count data [40]. To account for the non-independence of the actions of a territory-holding male and female, as well as any effects related to the use of stimulus model pairs in more than one replicate, ‘breeding pair/territory ID’ and ‘model pair ID’ were added as random effects (as per [41]). We then proceeded with stepwise refits of the model, each time without its least significant, highest order interaction term, using  $p = 0.05$  as the cut-off point. We used R 3.2.2 software (R Development Core Team) for all analyses.

### Results

When we applied a generalized mixed model to assess the effects of the species and sex of the focal territory holders and the ‘breeder/non-breeder’ status of the model intruder on the rate of aggression, we found a significant interaction between focal species and sex ( $t_{219} = 6.38$ ,  $p < 0.001$ ): male *H. nicaraguensis* exhibited a higher rate of aggression than females, whereas there was no pronounced sex difference in *A. sagittae* (Fig. 2). The effect of intruder status (i.e. breeder versus non-breeder colour markings) was not significant ( $t_{219} = 1.05$ ,  $p = 0.29$ ) (Fig. 2). We also considered the possibility that our results might have been affected by the artificial manipulation of our models (i.e. image manipulation in Photoshop). We assessed this by reanalysing the data comparing only the aggressive responses towards *A. astorquii* models that exhibited natural (i.e. non-manipulated) breeding versus non-breeding colour patterns. The results, however, remained qualitatively the same: there was an interaction between sex and species ( $t_{107} = 4.20$ ,  $p < 0.001$ ), whereas the status of the model (i.e. breeder versus non-breeder) did not have a significant effect ( $t_{107} = 0.818$ ,  $p = 0.41$ ).

## Discussion

We found that neither *H. nicaraguensis* nor *A. sagittae* territory holders reacted differently to breeder vs. non-breeder model intruders of *A. astorquii*. In other words, contrary to our expectation, neither of the two focal species adjusted their aggression to the allopatric heterospecific signal. Earlier studies using both manipulated [19] and natural [22] stimuli have nevertheless strongly indicated that *Amphilophus* cichlids do react differently to sympatric breeders and non-breeders, with coloration (of model intruders) being a sufficient cue for aggression level adjustments in both of our focal species [18, 19, 38]. Below we discuss why we did not find adjustment of aggression towards the different models of *A. astorquii* intruders in the current study.

First, we consider the possibility that one or both focal species had the capacity to correctly distinguish between breeder and non-breeder (model) intruders but chose not to modify their aggression because the signal was not clear or sufficiently relevant to induce a response. In the case of *H. nicaraguensis*, it remains possible, for example, that differences in the motivations of intruders of the more distantly related *Amphilophus*, as displayed by the breeding and non-breeding colour patterns, are not relevant enough for territory holders to significantly adjust their aggressive behaviour. However, this possibility is less likely to explain why the closely related and phenotypically similar *A. sagittae* territory holders also did not respond differently to the two breeder types, even though they do direct more aggression towards models of breeders compared to non-breeders of the sympatric *A. xiloaensis* [19]. It is nevertheless feasible that to avoid any costs of misplaced aggression more generally, both species may have evolved, or territory holders may have learned, not to modify their territorial aggression when the stimulus cues do not match well enough with the specific signals that are displayed by conspecifics or phenotypically similar species with which they are sympatric. Next, we consider proximate mechanisms that could have resulted in the lack of response to the allopatric breeding status signal. In other words, we consider the possibility that the territory holders might simply have not succeeded in making the distinction between breeders and non-breeders when these were allopatric.

At the proximate level, it is feasible that mere differences in markings and colour brightness between allopatric breeders and non-breeders, without any supporting behavioural differences, may have given too subtle a cue for the territory holders to adjust their aggression. In other words, because the ability to distinguish between breeders and non-breeders [19, 22] has, by default, evolved in interaction with species sharing the same environment (i.e. sympatric species), the territory holders may not be able to recognise the equivalent cues when signalled by allopatric species. This possibility supports the hypothesis and

empirical observations that interactions with non-native competitors or predators can result in inappropriate behavioural responses [27, 42–44]. For instance, the results reported in the current study are consistent with an earlier study investigating the response of *Amphilophus zalius* parents towards an introduced predator, the bigmouth sleeper (*Gobiomorus dormitor*) in Lake Apoyo [27]. That study showed that fry-guarding parents allowed the non-native predator to venture much more closely to their fry before reacting to them compared to the distance that native fish predators were allowed to approach. Hence, signals used to recognise competitors or predators may result in inappropriate behavioural responses when individuals are exposed to novel or unfamiliar signals, which can have negative fitness consequences for the receiver and/or benefit the novel (invasive) species [42, 45]. In this respect, if our results are due to a failure of the focal Lake Xiloá residents in recognising the breeding status signal of allopatric *A. astorquii* intruders, we do not currently know whether the observed response would have been an overreaction to breeders or an underreaction towards non-breeders. In the case of an actual invader, a likely consequence of the former would be increased energy expenditure, whereas the latter could result in increased rates of predation on eggs and juveniles (see [27, 44]).

Finally, we consider the possibility that our models simply did not accurately represent differences between *A. astorquii* breeders vs. non-breeders. In this respect, we prepared our models by adjusting shading and contrast of one model in each pair to mimic the patterns of the opposite breeding status. However, we do not believe that this artificial manipulation of colour patterns per se explains the results. This is because even when we analysed the reactions towards *A. astorquii* models with natural breeding and non-breeding coloration, we still found no difference in response towards the two colour types. Furthermore, our models were thinner than actual fish, and we therefore cannot rule out the possibility that territory holders may have perceived the models as individuals in poor body condition. If this was the case, territory holders might have regarded the models as a lower threat compared to living intruders in good condition. However, it is important to point out that earlier studies have demonstrated significant aggression adjustments to colour differences in similar intruder models (i.e. with a thickness of 6 mm) of sympatric species [18, 19, 38].

Due to the argument of Ord et al. [5] that sex differences in the opportunity or ability to learn relevant cues may induce differences in heterospecific recognition between males and females, we also assessed differences between the sexes in their reactions towards breeder versus non-breeder models, when deliberately controlling for learning opportunities by using an allopatric stimulus. Our results do not provide evidence for sex differences in

recognition (or relevance) of the allopatric signal. We did nevertheless find an overall sex difference in aggressiveness in *H. nicaraguensis* but not in *A. sagittae*. This result is likely to reflect a general difference in sex roles between these two species. In particular, it seems that in *H. nicaraguensis*, more so than in *A. sagittae*, males and females have evolved divergent roles in territory defence. Specifically, we found that *H. nicaraguensis* males were far more aggressive compared to females, whereas aggressive responses were much more evenly distributed between the sexes in *A. sagittae*. We note that the size of our intruder models (total length: 16 cm) relative to self may have been perceived more similarly between the sexes in *A. sagittae* as compared to *H. nicaraguensis*, given that males are only slightly larger than females in the former (typical male standard length: 13–18 cm, typical female standard length: 10–15 cm) but much larger than females in the latter (typical male standard length: 8–12 cm, typical female standard length 4–8 cm) [25, 29].

## Conclusion

We found that although the two focal species share the same breeding habitat and are likely to be subject to similar ecological pressures in the shared environment, they nevertheless exhibited different sex roles in breeding territory defence. This means that different species have evolved divergent approaches for successful parental care. However, we did not find evidence for differentiation between sexes in the pattern of aggression adjustment in either species. Interestingly, we found that when the stimulus was allopatric, aggressive defence of the breeding territory was not adjusted towards stimuli with contrasting breeding status coloration, independent of the phenotypic similarity between the territory holders and intruders. This is in contrast to earlier studies that used similar methodology but with sympatric (rather than allopatric) intruder stimuli. When considered together with these earlier findings, the current results underscore the importance of considering familiarity and coevolution in heterospecific competitor recognition.

## Ethics

The study was approved by MARENA (Ministerio del Ambiente y los Recursos Naturales, Nicaragua: permit no. 013-102013) and is compliant with all relevant laws for the ethical treatment of animals in scientific research.

## Availability of supporting data

Our data have been uploaded to Dryad: <http://dx.doi.org/10.5061/dryad.79p2b>.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

TKL conceived the study, and had the leading role in designing the study, performing the experiment, and writing the manuscript. BMW and WS provided ideas for the study design and, together with KG, contributed to preparations for the experiment. KG participated in gathering the data, and together with TKL, analysed the data. All authors played a role in preparing the manuscript and approved the final version of it.

## Acknowledgments

We thank Marta Barluenga, Lasse and Pirjo Lehtonen, Ken McKaye, Mark McKaye, Eric van den Berghe, and Jeffrey McCrary for logistic support. Funding was provided by the Department of Biology at the University of Turku, the Holsworth Wildlife Endowment Fund, and the Linnean Society of New South Wales.

Received: 26 June 2015 Accepted: 14 December 2015

Published online: 04 January 2016

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