Know thine enemy: intrasexual selection and sympatric speciation in Lake Victoria cichlid fish
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# Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 1</td>
<td>General Introduction</td>
<td>7</td>
</tr>
<tr>
<td>Chapter 2</td>
<td>Can male-male competition stabilize speciation? A test in Lake Victoria haplochromine cichlid fish</td>
<td>23</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>Male-male competition and speciation: aggression bias towards differently coloured rivals varies between stages of speciation in a Lake Victoria cichlid species complex</td>
<td>39</td>
</tr>
<tr>
<td>Chapter 4</td>
<td>Direct male-male competition can facilitate invasion of new color types in Lake Victoria cichlids</td>
<td>51</td>
</tr>
<tr>
<td>Chapter 5</td>
<td>Learned aggression biases in Lake Victoria cichlid fish</td>
<td>65</td>
</tr>
<tr>
<td>Chapter 6</td>
<td>Male-male competition in single-color and mixed-color assemblages of cichlid fish: understanding species coexistence and diversification via intrasexual selection</td>
<td>75</td>
</tr>
<tr>
<td>Chapter 7</td>
<td>Territory quality affects female preference for male nuptial coloration in a Lake Victoria cichlid fish</td>
<td>89</td>
</tr>
<tr>
<td>Chapter 8</td>
<td>Social stimulation, nuptial coloration, androgens and immunocompetence in a sexual dimorphic cichlid fish</td>
<td>105</td>
</tr>
<tr>
<td>Chapter 9</td>
<td>Female-female aggression can promote stable color polymorphism</td>
<td>121</td>
</tr>
<tr>
<td>Chapter 10</td>
<td>General Discussion</td>
<td>135</td>
</tr>
</tbody>
</table>

Nederlandse Samenvatting 147

References 159

Dankwoord 175

Addresses of co-authors 178
1

General Introduction

Peter D. Dijkstra
Understanding the forces that generate and maintain biodiversity

Understanding the mechanisms that generate and maintain biological diversity is of central importance in evolutionary biology (Ricklefs & Schluter 1993). Darwin (1859) believed that natural selection could produce two new species if intraspecific competition erodes less fit intermediate phenotypes. This view receded during the Modern Synthesis in the mid 1900s, when the focus of speciation research shifted away from natural selection as the driving force towards the role of biogeography in limiting gene flow (Dobzhansky 1937; Mayr 1942). The empirical basis for this viewpoint is the observation that sister species tend to be geographically isolated. In geographically isolated populations, reproductive isolation is thought to accumulate as a byproduct of independent evolution through the establishment of incompatibility alleles. This may lead to pre-mating isolation as a result of incompatible mating systems between diverging populations (e.g. Mayr 1963; Dodd 1989), or post-mating isolation when interbreeding results in inviable, sterile (e.g. for reviews: Rice & Hostert 1993; Wu & Ting 2004) or less well adapted offspring (Rice & Hostert 1993; Schluter 2000; Coyne & Orr 2004). In the geographical view populations ‘within cruising range’ of one another cannot escape from the homogenizing effects of gene flow, whereas geographically isolated (allopatric) populations can diverge freely through (for example) genetic drift (Mayr 1963). Sympatric speciation, that is the formation of species within a freely interbreeding population, has therefore been a highly controversial idea.

The allopatric view was often forcefully defended with some proponents of the allopatric speciation model actively discouraging the study of sympatric speciation (Mayr 1963; Maynard Smith 1966; Futuyma & Mayer 1980; Felsenstein 1981). The allopatric speciation model is powerful, because the essence of speciation, that is reproductive isolation, is guaranteed externally by geography, providing assortative mating essentially for free (Kirkpatrick & Ravigné 2002) and allowing populations to diverge by virtually any force. Sympatric speciation is theoretically more difficult to achieve, because the evolution of reproductive barriers is caused not by geography, but by biological attributes of organisms (Coyne & Orr 2004).

Selection and sympatric speciation

In the past decades growing theoretical and empirical support for the likelihood of sympatric speciation emerged, and for putative mechanisms that might underlie its occurrence (for reviews see Schluter 2001; Turelli 2001; Via 2001; Kirkpatrick & Ravigné 2002; Coyne & Orr 2004; Rundle & Nosil 2005). In 1988 it was verified that the first described case of sympatric speciation, two host races of *Rhagoletis pomonella*, which were formed by adaptation to different host plants (Bush 1969, explained below), were genetically differentiated (Feder et al. 1988; McPheron et al. 1988). Rice & Hostert’s (1993) influential review of laboratory studies of speciation
provided strong empirical evidence that reproductive isolation can evolve at least in a laboratory setting even without pre-existing barriers to gene-flow. In addition, a number of mathematical models now illustrates the plausibility of sympatric speciation (e.g. Van Doorn et al. 1998; Higashi et al. 1999, Dieckmann & Doebelli 1999; Van Doorn & Weissing 2001; for reviews see Turelli et al. 2001; Via 2001). This has led to an increase in interest in sympatric speciation, reflecting a resurgence of Darwins’ original views of adaptation and speciation (Schilthuizen 2000). Since sympatric speciation models traditionally rely on selection as a cause of divergence, attention shifted back from speciation modes based on geography towards understanding how selection might directly contribute to the emergence of new species (Via 2001; Kirkpatrick & Ravigné 2002). The shift in focus of speciation research is illustrated by Kirkpatrick & Ravigné’s (2002) new classification of speciation by selection. In their view, allopatry and sympathy do not need to be treated as qualitatively distinct situation, because one could treat geography as simply another form of assortative mating, just like animals may mate assortatively as result of phenotypic variation in habitat choice or timing of reproduction. None the less, geography is still important in understanding speciation, because it affects the ecological sources of divergent selection that can act, as well as the possibilities of gene-flow between populations (Rundle & Nosil 2005). In fact, speciation may often occur between the extremes of sympatric and allopatric speciation, with often a parapatric stage in which gene-flow can be reduced by distance (see i.e. Arnegard et al. 1999; Streelman & Danley 2003).

Speciation by divergent natural selection is now widely acknowledged as a major force in speciation, with or without geographic barriers (Rundle & Nosil 2005). In the classical scenario of sympatric speciation, competition for diverse resources may generate disruptive frequency-dependent natural selection, leading to splitting of two new species. A disruptive selection regime, that is selection favoring the extreme ranges of the phenotypic distribution, is maintained by frequency-dependent selection. Frequency-dependent selection is selection that ensures stable co-existence of more than one phenotype by favoring rare phenotypes. Individuals with intermediate phenotypes are outcompeted by those with well-adapted extreme phenotypes, leading to the evolution of nonrandom mating based on traits under direct selection (via pleiotropy of physical linkage) or due to recruitment of other isolating traits through development of genetic correlations (via linkage disequilibrium) (Dieckmann & Doebeli 1999; Kondrashov & Kondrashov 1999). In this view, barriers to gene flow evolve between populations as a result of divergent ecological selection, and nonrandom mating only plays a secondary role (Maynard Smith 1966; Bush 1969; Kondrashov & Mina 1986; Filchak et al. 2000). Laboratory experiments have shown that ecological speciation can occur, since reproductive isolation evolved as a correlated response of adaptation to different environments in experimental studies (reviewed in Rice & Hostert 1993). There is also compelling evidence for its operation in nature (reviewed in Schluter 2001; Coyne & Orr 2004). The apple maggot fly, *Rhagoletis*
**pomonella**, is a classical example of ecologically driven sympatric speciation by host-related adaptation resulting in virtually complete premating isolation among different species occupying different host plants (Filchak et al. 2000). Other examples for sympatric speciation are limnetic and benthic morphs of Arctic charr, *Salvelinus alpinus*, from Lake Galtábol in Iceland (Gíslason et al. 1999), and the cichlids from small crater lakes in Nicaragua and Cameroon (Schliewen 1994; Barluenga et al. 2006). In these cases, evidence suggests sympatric speciation leading to eco-morphologically distinct, yet genetically monophyletic, morphs or species.

**The role of sexual selection**

Sexual selection, that is nonrandom mating leading to differential mating success of different genotypes, has also been recognized as a powerful force that can drive speciation (e.g. Darwin 1871; Lande 1981; West-Eberhard 1983; Civetta & Singh 1999). This view is supported by comparative studies that indicate that recently diverged species exhibit only subtle eco-morphological differentiation but striking divergence in their secondary sexual characters (Dominey 1984; Eberhard 1985; Wilson et al. 2000). DNA sequence analysis has revealed an extraordinary divergence of sex-related genes, particularly between closely related taxa (e.g. Vacquier 1998; Wyckoff et al. 2000), lending some support for the hypothesis that sexual selection has acted on these genes during speciation. A number of studies have indicated a correlation between the intensity of sexual selection and species richness (Barraclough et al. 1995; Mitra et al. 1996; Owens et al. 1999; Stuart-Fox & Owens 2003). Mitra et al. (1996) showed that bird groups with promiscuous mating systems (a presumed proxy for sexual selection) are more species-rich than non-promiscuous sister groups. Also, from a theoretical point of view, sexual selection is a powerful means to establish reproductive isolation, as it directly influences the mating structure (e.g. Kirkpatrick & Ravigné 2002) and can rapidly create a correlation between preference and male trait. Because attractive fathers produce attractive sons with high mating success (Fisher 1930), mate choice would cause a rapid build-up of a correlation between preference genes and male trait genes in a self-reinforcing manner, a process also known as the Fisherian runaway process (Fisher 1930; Lande 1981; Kirkpatrick 1982). According to Coyne & Orr (2004), the observation that sexual selection alone, unaided by ecology, appears to drive speciation is ‘one of the most important findings in the last decade of work on speciation’ (p 407).

Theoretical biologists have indicated the plausibility of sexual selection driving sympatric branching without the aid of natural selection. If mate choice depends on female preference for variants of male display, a population exhibiting enough variation may split apart via assortative mating, leading to divergent Fisherian runaway processes (Turner & Burrows 1995; Payne & Krakauer 1997; Higashi et al. 1999; Takimoto et al. 2000; Van Doorn et al. 1998; Van Doorn et al. 2004). Sexual selection may lead to reinforcement of reproductive isolation by
effectively eliminating intermediate phenotypes due to reduced mating success. Evidence of reduced mating success in hybrids was found in hybrids from pairs of butterfly species (Davies et al. 1997; Naisbit et al. 2001), from limnetic and benthic stickleback forms (Vamosi & Schluter 1999), and haplochromine cichlid fish (Stelkens & Seehausen in prep.). Several cases in nature exist in which authors implicated sexual selection in sympathy (see Panhuis et al. 2001). One of the best examples are the impressive radiations of haplochromine cichlid fish in Lakes Victoria and Malawi in Africa (Kocher 2004). Their staggering diversity has motivated many models of sympatric speciation by disruptive sexual selection (Turner & Burrows 1995; Higashi et al. 1999; Arnegard & Kondrashov 2004; Van Doorn et al. 2004). This is because of the monophyly of groups within single lakes (Lake Victoria: Nagl et al. 2000; Verheyen et al. 2003; Seehausen et al. 2003, Lake Malawi: Moran et al. 1994; Kocher et al. 1995; Albertson et al. 1999), and the difficulty to envision sufficient geographic barriers within lakes to allow the formation of hundreds of species in less than 2 million years (Lake Malawi) or even less than 15,000 years (Lake Victoria). Closely related sympatric cichlid species are anatomically and ecologically similar, while exhibiting striking variation in male nuptial coloration (Seehausen 1996; Seehausen & Van Alphen 1999), with this variation affecting mate choice (Seehausen & Van Alphen 1998; Knight & Turner 2004). Also the mating system is conducive to extremely strong sexual selection, with lekking males and exclusive female parental care in the form of mouthbrooding (Kornfield & Smith 2000).

The problems in sympatric speciation by sexual selection alone

Recent theoretical models indicate that sympatric speciation by sexual selection alone requires extremely restrictive conditions (Arnegard & Kondrashov 2004; Kirkpatrick & Nuismer 2004; Van Doorn et al. 2004; Gavrilets 2004). Some even suggest that the process is theoretically impossible (Arnegard & Kondrashov 2004). In the traditional models of sympatric speciation by sexual selection, disruptive selection by female preferences for opposite extreme male displays may lead to assortative mating and can split a population apart (Turner & Burrows 1995; Higashi et al. 1999; Takimoto et al. 2000). Considerable genetic variation is required to trigger divergent Fisherian runaway processes (Higashi et al. 1999). Sexual selection, however, may rapidly deplete genetic variability in female mating preferences, hereby constraining sympatric speciation (e.g. Kirkpatrick & Nuismer 2004). Another problem is that speciation can only occur from a rather narrow range of initial phenotypic distribution of female preference and male display, which must be close to symmetric (Arnegard & Kondrashov 2004; Van Doorn et al. 2004). When relaxing this initial symmetry, disruptive sexual selection destabilizes the population, pulling it in the direction of one phenotypic extreme.

These problems may be illustrated as follows. Let us assume a population with blue males, and females basing their choice on male nuptial hue. A novel, say red male phenotype, arises in the population. Since female mating preference and
male trait are likely to co-evolve, it seems unlikely that genetic variation in female mating preference is sufficiently large enough to allow for invasion of a red male. The novel, red male requires an advantage to invade to overcome the pre-existing female mating preference for the already established blue trait. A second problem is the stable co-existence of red and blue morphs, both during and after speciation. If slightly more females prefer blue, the whole population becomes blue, and red is driven to extinction. Both problems can be resolved by negative frequency-dependent selection, i.e. advantage to rarity. In the classical scenario of sympatric speciation by disruptive natural selection, this frequency-dependence is generated by divergent ecological selection. Such selection may confer an advantage to rare ecotypes that occupy a less crowded niche, and may lead to ecological differentiation that forms the basis of long-term co-existence of incipient species (Van Doorn et al. 2004).

Male-male competition is an essential ingredient for sympatric speciation by sexual selection

Sufficient genetic variation in female mate choice has to be present in order to trigger divergent runaway processes. Genetic variation in female mate choice could be maintained by disruptive frequency-dependent selection on female mate choice by female-female competition for males (Van Doorn et al. 2004; also see Lande 2001; Almeida & Vistulo de Abreu 2003). Female-female competition for males may come about when we assume that males cannot father an unlimited number of offspring, due to time and energy constraints or sperm depletion. If this is the case, the female preference immediately becomes dependent on the strategies of other females in the population, rendering female preference frequency-dependent. Even when this condition was met, simulations indicated that simultaneous splitting of female mating preference and male trait was not possible (Van Doorn et al. 2004). This was due to the fact that males benefit from mating as frequently as possible, whereas females benefit when they find a mate that has not mated before. This conflict between the sexes translates into opposing selective forces: when it pays for males to undergo branching, the females will experience stabilizing selection and vice versa. In other words, in case the female mating preference distribution is wider than the male trait distribution, males experience disruptive selection as a result of selection favoring males to opt for extreme female preference types, because most matings for males can be obtained in the tails of female mating preference distribution. At the same time, females will then experience stabilizing selection, moving the female mating preference towards the centre of the distribution, because when the male trait is subject to disruptive selection, females should prefer male types in the centre as these are least mated. The opposite is the case when the male trait distribution is wider than the female mating distribution. Thus, either one of the sexes can undergo branching, but simultaneous branching is not possible.
For these reasons, additional sources of disruptive selection are required to make sympatric speciation possible, separate from disruptive selection already generated by mate choice and female-female competition. Such selection could be caused by a variety of mechanisms (Weissing 1996), and any process that leads to an intrinsic advantage of rarity of either rare female preference types, and/or rare male traits is adequate (Van Doorn et al. 2004). Since in several taxa male-male competition is a major component of sexual selection (Andersson 1994), one concrete example is selection on the male traits involved in mate choice via aggressive male-male competition for breeding sites. This is well known for sticklebacks, where the red breeding coloration is important in female mate choice (Milinski & Bakker 1990) and in agonistic interactions (Bakker & Milinski 1993; Rowland et al. 1995). Haplochromine males typically flush their colors used in interspecific mate choice when confronted by rival males. The possibility that male-male competition can generate negative-frequency-dependent selection and thereby facilitate sympatric speciation forms a crucial background for my thesis. In haplochromines males, territory ownership is a prerequisite to gain access to spawnings (Parker & Kornfield 1996; Maan et al. 2004). Hence, competition over territory sites is intense, and is likely to affect sexual selection. The mechanisms that may generate or contribute to rare male advantages in male-male competition will be now discussed.

(1) Own-type bias in aggression
If territorial males direct more aggression to rivals that phenotypically resemble themselves than to different phenotypes, rare male varieties would enjoy a frequency-dependent advantage because they receive less aggression (Mikami et al. 2004; Seehausen & Schluter 2004). This might indeed turn into a fitness advantage. It could come about by rare males being able to obtain and defend high-quality territories, maintaining higher body condition, or spending less time on territorial defense, leaving more time and energy for male courtship. If female mate choice is not exclusively based on male color, but on a combination of male attributes, such as territory quality and courtship vigor, an own-type bias in aggression would result in rare male types being more likely to acquire a breeding territory and/or access to females than the common male type (Seehausen & Schluter 2004). The advantage would decrease with increasing frequency of the rare morph, due to increased intramorph encounters. An own-type bias in aggression thus facilitates the invasion of a novel male phenotype and stabilizes co-existence of incipient species.

(2) Dominance through color effects
Color effects on dominance may facilitate invasion of a novel male phenotype. Several studies (e.g. Hill & Barton 2005) found dominance effects of bright coloration on winning dyadic combats. This effect is often attributed to bright, for example red coloration, having an intrinsic intimidating value. Such color effects on dominance do not automatically lead to stable co-existence of incipient species,
unlike own-type biases in aggression. We need to invoke other mechanisms to generate frequency-dependent selection. A possible mechanism is behavioral adaptation, with the ancestral morph increasing the frequency of aggressive acts towards the novel morph once the latter is established in the population. A novel coloration may also confer an advantage when its novelty elicits a fear response in the ancestral morph. Frequency-dependence may arise when the degree of fear response declines with increasing frequency of the novel morph, due to males becoming accustomed to a novel color.

(3) Dominance through elevated aggression level
The novel morph may be intrinsically more aggressive than the resident morph. This mechanism requires a pre-existing connection between color and elevated aggression level (Fernald 1976; see also Lazarus & Crook 1973). For example, an endocrinological change inducing the novel color (more androgens results in emergence of a novel, brighter color) could have pleiotropic effects on aggression (more androgens result in elevated aggression level). Frequency-dependence may arise when with increasing relative frequency of the novel color morph, they become more frequently involved in more aggressive and hence more costly intramorph conflicts than the ancestral morph. This exhaustion effect of the novel males may then mute their behavioral dominance over the ancestral males.

This thesis
Speciation by sexual selection research has traditionally concentrated on mechanisms for divergence driven via female mate choice (intersexual selection). The pivotal role of competition between members of the same sex (intrasexual selection) has been largely overlooked.

In this thesis, I describe a series of experimental studies investigating the role of intrasexual selection in sympatric divergence, using Lake Victoria haplochromine cichlid fish as a model system. Such experiments are needed, as only indirect or descriptive support exists for the hypothesis of male-male competition facilitating sympatric divergence. I also investigate color expression as a result of male-male competition, and ask if the intensity of sexual signaling provides information about the genetic quality of the bearer. Speciation theory suggests that ‘good genes’ mechanism of female mate choice are less likely to drive (sympatric) speciation than purely divergent Fisherian runaway selection (Lande 1981; Payne & Krakauer 1997; Turner & Burrows 1995; Van Doorn et al. 1998; 2004). Under Fisherian runaway selection, population bifurcation may proceed in arbitrary directions, whereas ‘good genes’ sexual selection is less arbitrary, leading to the evolution of only those male traits that reliably indicate genetic quality. Identifying the mechanisms that cause sexual selection in haplochromines has important implications for our understanding of sympatric speciation.

The project is part of a larger research program funded by the Netherlands Organisation for Scientific Research (NWO), entitled ‘Functions and Mechanisms
of Sexual Selection and The Sympatric Origin of African Cichlid Fish’, involving in three PhD projects. The program investigates many facets important in the process of sympatric speciation by disruptive sexual selection. Inke van der Versluijs concentrates on ‘The origin and maintenance of covariance between genes for mate preference and genes for coloration during sympatric speciation by sexual selection in cichlid fish’ (University of Leiden, supervision Prof. Ole Seehausen and Prof. Jacques van Alphen). Machteld Verzijden focuses on ‘The development of sexual preferences in rapidly evolving cichlid fish’ (University of Leiden, supervision Prof. Carel ten Cate). The project had further close association with two other PhD projects dealing with sexual selection in Lake Victoria cichlid fish. Martine Maan worked on ‘Sexual selection and speciation. Mechanisms in Lake Victoria cichlid fish’ (University of Leiden, defended in May 2006, supervision Ole Seehausen and Jacques van Alphen). Michele Pierotti works on ‘Speciation and sex determination in color polymorphic cichlid species from Lake Malawi and Lake Victoria’ (University of Hull, supervision Ole Seehausen).

Haplochromine cichlid species flock: model system for sympatric speciation by sexual selection

Fishes of the family *Cichlidae*, distributed from Central and South America, across Africa to Madagascar and India, represent the most species-rich family of vertebrates. It is the rapid radiation of cichlid fish species in the Great Lakes of East Africa that has attracted much attention (e.g. Fryer & Iles 1972; Echelle & Kornfield 1984; Meyer 1993; Seehausen 1996; Barlow 2000; Seehausen 2000; Danley & Kocher 2001; Kocher 2004; Genner & Turner 2005; Seehausen 2006a). In each of the major lakes (figure 1), a few species have initiated the rapid adaptive radiations. Lake Tanganyika has a species flock of at least 197 endemic cichlids (Poll 1986), whereas Lake Malawi and Lake Victoria each contain more than 500 species (Turner et al. 2001). While these radiations were once thought to reflect allopatric speciation (Mayr 1963; Fryer & Iles 1972), many now consider it the result of both allopatric and sympatric speciation.

The cichlids are popular in speciation research, mainly due to their amazingly fast speciation rates (e.g. Won et al. 2005). Selection and phylogenetic signals of population bifurcation is usually rapidly lost after speciation, rendering currently speciating lineages like the haplochromines excellent model systems to test assumptions involved in speciation processes. Each lake contains a vast number of recently diverged species, as well color polymorphisms within species, representing different stages of speciation (Seehausen 1997; Seehausen et al. 1999a; 1999b). Next to biogeographical factors, several biological attributes of haplochromines have promoted their diversification, such as dispersability, ecological adaptation and sexual selection, and extinction as a diversity limiting process (Seehausen 1999).

Earlier models emphasized vicariant events resulting from changes in lake water levels in generating the radiation in East African Lakes. Vicariance is the
evolution of reproductive isolation after the splitting of the geographic ranges of a species into reproductively isolated populations. In Lake Tanganyika dramatic changes in water levels, with subsequent fragmentation of the lake and allopatric divergence during dry events, is reflected in the phylogeographic patterns of Lake Tanganyika’s rock-dwelling cichlids (Sturmbauer & Meyer 1993). The effects of variance are not universal. It is not detectable in all taxa in Lake Tanganyika (Meyer et al. 1996) and the basin morphologies of Lake Malawi and Lake Victoria do not generate multiple basins during low water stands (Scholz & Rosendahl 1988). It is therefore likely that the vast majority of species have evolved in situ in all three lakes (Meyer 1993; Moran et al. 1994; Nagl et al. 2000). It appears that ecological and behavioral factors have the largest effects on the diversification of cichlids. One aspect that could have influenced this diversification is their dispersal behavior, which determines the levels of gene-flow between groups and hence their potential to diverge. Recent estimates of current levels of gene-flow in Lake Malawi mbuna, however, indicate very restricted migration over limited geographical scales. (Van Oppen et al 1997; Arnegard 1999; Markert et al. 1999; Danley et al. 2000). Danley & Kocher (2001) suggest that the levels of gene flow between communities of cichlids, often occurring at a single island or headland, declined since the colonization of Lake Malawi. Given the enormous size of the Great African Lakes, both sympatric speciation within a locality, and allopatric speciation between localities should have been important. Seehausen & Van Alphen (1999) plotted the number of sister species pairs against the distributional overlap between the species, and identified a bimodal distribution, with modes on allopatric and sympatric. The proportion of sister species pairs that are different in body color was much higher in sympatry than in allopatri. These findings suggest that not only allopatric speciation but also sympatric speciation by disruptive sexual selection on color polymorphism is a common mode of speciation in Lake Victoria cichlid fish.

Figure 1 The three Great Lakes of East-Africa. Courtesy of M. Maan and M. Brittijn
Both sexual and natural selection have played a major role in the radiation of haplochromines. Cichlids have evolved numerous trophic adaptations for food acquisition. Feeding guilds include rock scrapers, predators on eggs and young, bottom feeders, pelagic zooplanktivores, molluscivores, and even fin, scale and eye biters (Barlow 2000). It has been suggested that the flexibility of the pharyngeal jaw system has contributed to the rapid adaptive radiation of haplochromines (Liem 1974; Galis & Drucker 1996; Albertson et al. 2003). Ecological selection likely precipitated the large scale ecological divergence into different genera (Danley & Kocher 2001).

**Sexual selection in haplochromines**

Within genera, many closely related species differ very little in diet, breeding site, and habitat use, while these species exhibit large variation in male nuptial coloration. This variation in color is important in mate choice by female mating preferences (Seehausen & Van Alphen 1998; Knight & Turner 2004), and also important for biologists to distinguish congeners. This has lead to the hypothesis that sexual selection was the main engine of speciation in these groups (Dominey 1984; Seehausen 2000). This is particularly true for the rock-dwelling cichlids in Lake Malawi, the *mbuna* (Genner & Turner 2005), and a similar color-rich group in Lake Victoria, the *mbipi* (Seehausen 1996).

In the rock-dwelling cichlid communities in both lakes, two types of color polymorphisms are common among closely related sympatric species or morphs (Seehausen et al. 1999a). One is the blue red-yellow polymorphism. A previously well studied example is the sympatric sibling species pair, *P. nyererei* and *P. Pundamilia* from Lake Victoria (Seehausen 1997; Seehausen & Van Alphen 1998; Haesler & Seehausen 2005), or the *Pseudotropheus* complex from Lake Malawi (Konings 2001; Smith & Kornfield 2002). The other is the blotch polymorphism characterized by plain morphs, and morphs carrying melanin blotches on a white or orange background, with homozygotes being completely black, white or orange (Seehausen 1996; Konings 2001). The blotch polymorphism is associated with sex determining genes (Seehausen et al. 1999b; see also table 1 in Lande et al. 2001). Mate choice experiments have shown that the color polymorphisms are associated with female and male mating preferences (Seehausen & Van Alphen 1998; Seehausen et al. 1999b; Knight & Turner 2004).

The breeding system and sexual dimorphism of rock-dwelling cichlids is conducive to strong sexual selection. Males do not participate in parental care, whereas females provide care in the form of mouthbrooding for several weeks (Seehausen 1996; Genner & Turner 2005). The females also protect the fry after release in Lake Victoria cichlids. Sexual dimorphism is often very pronounced, with males usually attaining a larger size than females, and displaying bright coloration, and females that are often very cryptic (Dominey 1984). Males often form leks where they defend individual territories. Usually, only territorial males fully express their nuptial coloration, and males readily flush their colors upon exposure.
to a rival male. Females visit leks for spawning, and often mate with several males (Kellog et al. 1995; Parker & Kornfield 1996; Maan et al. 2004). Territory ownership is prerequisite to gain access to spawnings, and alternative mating tactics such as ‘sneaking’ are probably not very important in haplochromines (Parker & Kornfield 1996; Maan et al. 2004). Many sexually mature males cannot establish territories, and even among territorial males reproductive success is skewed towards a few lucky males (Kornfield & Smith 2000). Male-male combat competition is severe, as has been indicated by removal experiments that lead to intense disputes between many (non)territorial males trying to take over the vacant territory (Sharp 1981, Hert 1990; 1992; 1995; Dijkstra unpublished). Importantly, the quality of the territory may influence female mate choice. Females of sandy shore cichlids of Lake Malawi prefer males occupying a territory that is central in the lek arena (McKay 1991; Kellogg et al. 2000), and for their large (McKay et al. 1990) or symmetrical bowers (Taylor et al. 1998). In *P. nyererei* males from Lake Victoria the size of a male’s territory influenced mate choice (Maan et al. 2004). The breeding system provides the raw material for intense inter- and intrasexual selection on male coloration.

In many species food control is probably only a secondary benefit of territorial behavior. This is also indicated by *P. nyererei* males foraging in shoals away from their territories. In algae-scraping species food control is more important, with both males and females defending territories to secure the algal mat (Ribbink et al. 1983; Seehausen 1996; Maan 2006). In this case females are less territorial than males (Maan 2006) and do not to occupy long-term territories like males. Haplochromine females become territorial in the mouthbrooding phase to stand guard over their fry and allow their young to return to their mother’s mouth when threatened by predators (Seehausen 1996). Thus, territorial behavior is likely to affect fitness in both male and female haplochromines.

**Evidence of male-male competition acting as a diversification force**

The role of male-male competition in facilitating sympatric speciation by sexual selection has not received much attention (Seehausen & Schluter 2004; Van Doorn et al. 2004). Male-male competition is widely viewed as being an important component of sexual selection (Andersson 1994; Wong & Candolin 2005). Some have postulated it could be an important agent of diversification. Here I will briefly summarize some of these studies.

First, West-Eberhart (1983) proposed such competition could drive allopatric speciation in taxa in which male-male competition is the dominant mechanism for sexual selection (see also Andersson 1994). For example, in some lizard genera it is thought that active female mate choice plays a relatively minor role, and that variation in reproductive success is largely the result of male-male aggression (Stuart-Fox & Owens 2003). Under this scenario it is plausible that local adaptation in the ornaments involved in male-male interactions may lead to ornament divergence and ultimately to allopatric speciation.
Secondly, in order to prevent erroneous aggression towards heterospecifics, it is beneficial to sympatric species to diverge in secondary sexual characters. Such a shift in secondary characters is referred to as character displacement between species driven by interspecific aggressive interactions (Alatalo 1994; Tynkkynen et al. 2004; 2006). Character displacement is often asymmetric in that interspecific aggression could drive the phenotype of the behaviorally subdominant species away of the behaviorally dominant species (Lorenz 1962; 1966; Alatalo 1994; Tynkkynen et al. 2004; 2006). In *Ficeluda* flycatchers interspecific aggression from collared flycatchers, *F. albicollis*, forces the similar black and white plumaged pied flycatcher, *F. hypoleuca*, males to nest in less preferred habitats. Collared flycatchers are less aggressive towards brown-colored heterospecifics (Saetre et al. 1993; Alatalo 1994). Probably as a consequence of increased aggression towards black and white males, there is character displacement in plumage coloration which is more divergent in sympatric than in allopatric populations (Alatalo et al. 1994). The idea of character displacement between species can be extended to sympatric speciation if we consider character displacement by male-male competition to take place within species rather than between species, promoting the emergence, persistence and divergence of a novel male phenotype in a population (Seehausen & Schluter 2004; Van Doorn et al. 2004).

As noted earlier, I distinguish three mechanisms by which male-male competition can contribute to the establishment of a novel male trait, and subsequent stabilization of preference and trait. These were (1) own-type bias in aggression, dominance through (2) color effects or (3) elevated aggression level. I will now describe for each mechanism the existing evidence.

(1) Own-type bias in aggression
There is indirect evidence that haplochromines males bias aggression towards rivals of their own color type. In many Lake Victoria cichlid communities, closely related species with different male nuptial coloration occurred more in sympatry than expected by chance. Also, closely related species of the same color type occurred less together than expected by chance (Seehausen & Schluter 2004). Although disruptive selection on male color by female mate choice predicts the first pattern (Seehausen et al. 1997), it does not predict the negative association between closely related species of the same color, whereas own-type biases in aggression do. Interestingly the pattern was also evident between different morphs belonging to the same species. In the same study it was found that territorial *Neochromis omnicaeruleus* and *N. rufocaudalis* (‘red tail’) males tend to have territorial neighbors of species that are different in nuptial coloration from themselves (Seehausen & Schluter 2004). Kohda (1998) showed in Lake Tanganyika that the territories of *Petrochromis polyodon* were separated to a greater degree among conspecific males than among heterospecific males. These patterns are consistent with the interpretation that individuals among closely related species bias aggression towards phenotypic similar species, exerting negative frequency-
dependent selection on male nuptial coloration, and causing character displacement both within and between species (Seehausen & Schluter 2004).

(2) Dominance through color effects
No such studies have been done with haplochromines, but two studies exist using American cichlid fish. In dyadic combats between Firemouth cichlids, *Cichlasoma meeki*, Evan & Norris (1996) showed that red coloration increased the probability to win. The same has been found for gold coloration in the Central American Midas cichlid, *Cichlasoma citrinellum* (Barlow 1983).

(3) Dominance through elevated aggression level
In haplochromine there are few or no reports on differences in aggression level between closely related species or morphs. In some Tanganyika cichlids, differences in aggression level have been found in association with alternative reproductive styles (e.g. Heg et al. 2005). In many animal species alternative morphs are associated with differences in aggression level/attack readiness, and/or success in aggressive interactions (e.g. Ruff: Lank et al. 1995; Lizards: Knapp & Moore 1996; Sinervo & Lively 1996; Midas cichlid: Barlow 1983; Swordtail fish: Heuts & Nijman 1998; Mosquitofish: Horth & Travis 2002; Gouldian finch: Pryke et al. 2006; for reviews Roulin 2004 [birds]; Gross 1996). Often, differences in male agonistic behavior between morphs are used to understand the evolution of alternative reproductive styles (Rubenstein 1980; Gross 1996). A commonly seen pattern is that one morph gains matings by defending a mating site/or females from other males, and the other one obtains matings by alternative means, such as ‘floating’ behavior (e.g. Knapp & Moore 1996; Sinervo & Lively 1996). For example, in the side-blotched lizard, *Uta stansburiana*, males display one of three alternative throat color morphs (orange, blue and yellow), each with an associated territorial behavior (Sinervo & Lively 1996; Sinervo & Clobert 2003); orange males are super-dominant and defend large territories, blue-throated males defend small territories, while the sneaking yellow males do not defend territories.

In conclusion, a substantial body of literature has focused on the role of territoriality and aggressive interactions in driving the evolution of color patterns, and in promoting the evolution of color polymorphisms associated with alternative reproductive styles. Few have embedded it in the context of sympatric speciation. Some of this evidence is consistent with the hypothesis that rare male advantages may emerge from intrasexual competition. Now, experimental tests are needed.
Outline of thesis and model species

In chapter 2-7 I studied two recently diverged, ecologically and anatomically similar sympatric cichlid species pairs consisting of *P. Pundamilia* (Seehausen et al. 1998a) with blue and *P. nyererei* (Witte-Maas & Witte 1985) with red nuptial coloration. In chapter 3 I also use *P. 'pink anal'* (Seehausen 1996) as a representative of a *Pundamilia* species with blue nuptial coloration. Red and blue phenotypes are anatomically similar forms that behave like reproductively isolated species in some locations, and like hybridizing incipient species in other locations (Seehausen 1996; Seehausen et al. 1997). Blue phenotypes have a lake-wide distribution whereas red phenotypes have a patchy distribution and always co-occur with blue phenotypes. The blue form has the highest record of sympatric occurrences with other members of the *Pundamilia* complex (Seehausen 1996; Seehausen & Van Alphen 1999). It seems hence likely that blue represents the ancestral state and that blue populations have been invaded repeatedly and independently by red morphs during speciation (Seehausen 1997; Seehausen & Van Alphen 1999; Seehausen & Schluter 2004). The different populations of *Pundamilia* were taken as different speciation stages, beginning with an entirely blue population where red males had not been able to gain a foothold yet, to populations where red and blue are hybridizing incipient species, and finally to populations where speciation has been completed with reproductively isolated red and blue species.

Own-type biases in aggression (chapter 2 and 3) and differences in aggression level (chapter 2) were measured in wild-caught *Pundamilia* males using a simulated intruder choice test. Territorial defenders were presented with red and blue stimulus fish, each enclosed in transparent tubes, mimicking intruding rival males, and recorded the aggressive response of the territorial defender. In chapter 2 light manipulations experiments revealed that aggression preferences are based on color differences, suggesting that color is not only important in mate choice, but also in intrasexual signaling. Following up to this chapter, in chapter 3 I tested in blue *Pundamilia* fish from six different populations whether aggression bias depends on speciation stage.

Color-effects on dominance can be measured by staging dyadic combats, mimicking two males disputing over a vacant territory. In chapter 4, I tested the hypothesis that red coloration confers an advantage in direct combat, assisting red phenotypes to invade. To this end, combats were staged between red and blue males under both white and green light condition that effectively eliminates the color difference.

How aggression biases come about in *Pundamilia* is unclear. Chapter 5 investigates the role of learning and genetics in shaping aggression biases in three populations of blue *Pundamilia* cichlid species. Males were given prolonged experience with red males or only blue males and subsequently subjected to a simulated intruder choice test.
In chapter 6 I experimentally tested in a group situation whether coexistence of red and blue *Pundamilia* phenotypes could be facilitated through male-male competition. The experiment allowed us to validate findings reported in chapter 3-6 under a more natural setting. We studied the formation of dominance hierarchies in assemblages composed of either only red males, only blue males, or males of both color types.

How are rare male advantages in male-male competition translated into fitness benefits? One possibility is that female mate choice is mediated by territory quality, and that territory quality has the potential to override the female mate preference for male nuptial coloration. Chapter 7 investigated this possibility using females of *Pundamilia nyererei*. Females could choose between a conspecific red male, and a heterospecific blue male. Territory quality was manipulated by varying tube size that males use as the centre of their territories.

In chapter 8 I tested the assumption that nuptial coloration is important in an intra-sexual context and may reveals aspects of male quality or ‘good genes’. Information about male quality may be relevant for both towards rival males in assessment of resource holding potential, and for prospect mates that might be interested in ‘good genes’. In *P. nyererei*, a cichlid fish with a carotenoid-based red breeding coloration, I tested the trade-off between color expression and one component of immune function.

In haplochromine cichlids female territoriality is likely to affect female fitness. Females aggressively fend off space during the mouth brooding phase to stand guard over their fry. In algae-scraping species, females occupy short term territories over rocky sites to secure the algal mat. Therefore, aggressive interactions between females too may promote invasion of novel color phenotypes, and species co-existence. In most haplochromine species, however, females of closely related species often look the same, providing less opportunity for color-based aggression biases. Yet, some conspicuous color phenotypes are found in females too. A good example is *Neochromis omnicaeruleus* (Seehausen et al. 1999b), a representative of the common blotched color polymorphism. The species consists of three distinct color morphs. In chapter 9 I tested whether an own-type bias exists in wild-caught female morphs of *Neochromis omnicaeruleus*. How the associated between color and aggression bias can be maintained in the face of gene-flow between morphs was also studied.

In chapter 10 the main findings are summarized and discussed in a more general context.
Can male-male competition stabilize speciation? A test in Lake Victoria haplochromine cichlid fish

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Abstract

It has been suggested that sympatric speciation can be driven by sexual selection on male mating traits alone. However, a fundamental problem for this process is the lack of ecological differentiation that would stabilize co-existence of incipient species through frequency-dependent selection. Such selection can also occur if male aggression is primarily directed towards similar rather than towards dissimilar phenotypes, so that rare male phenotypes would enjoy a negatively frequency-dependent fitness advantage. We experimentally tested such an aggression bias in two recently diverged, ecologically and anatomically similar sympatric cichlid species pairs of the genus *Pundamilia* from Lake Victoria. Territorial males of a pair of partially reproductively isolated species with red and blue nuptial coloration respectively, studied in the laboratory, were confronted simultaneously with both color types enclosed in transparent tubes. Red males were more aggressive to red stimuli under white light, but not when color differences were masked under green light. Blue males were equally aggressive to both stimuli in both light conditions. Males of two apparently fully reproductively isolated species, again one with red and one with blue nuptial coloration, studied in the field, both directed more aggressive behavior towards conspecific than towards heterospecific stimulus males. The differential allocation of aggression would create an advantage for males of the less abundant phenotype or species, thereby potentially supporting stable co-existence of the phenotypes. The finding that this effect was less clear in the partially reproductively isolated species pair than in the fully isolated species pair is discussed.
Introduction

Sympatric speciation, in which species arise without geographic isolation, is a much contested issue in evolutionary biology (Maynard Smith 1966; Felsenstein 1981; Bush 1994; Via 2001). There is theoretical and empirical evidence that sympatric speciation may occur in the wake of divergent ecological adaptation driven by competition for resources (Filchak et al. 2000; Schluter 2000; Turelli et al. 2001). In the traditional view, speciation is initiated by disruptive natural selection, although sexual selection by female mate choice on male ornaments may play a secondary role. Some models indicate that sympatric speciation can also be driven by sexual selection without disruptive natural selection (Lande & Kirkpatrick, 1986; Turner & Burrows 1995; Payne & Krakauer 1997; Higashi et al. 1999; Kawata & Yoshimura 2000; Van Doorn & Weissing 2001), or with a secondary role for the latter (Van Doorn et al. 1998). Under certain conditions, male trait and the corresponding female preference can jointly evolve by two runaway processes creating two reproductively isolated populations. Many of these models were inspired by the extraordinary diversity of cichlid fish in Lake Victoria and Lake Malawi in East Africa (Fryer & Iles 1972; Seehausen et al. 1997; 1999; Albertson et al. 1999). Sexual selection exerted by female preferences for male nuptial color patterns has been proposed to be an important mechanism underlying explosive speciation in these cichlids (Dominey 1984; Seehausen et al. 1997; Seehausen & Van Alphen 1999; Smith & Kornfield 2000; Kocher 2004). This hypothesis was based on the observation that closely related species in the most rapidly radiated groups display striking differences in nuptial coloration, while ecological and morphological differentiation among sister species is muted (Seehausen et al. 1998b; Genner et al. 1999a; Albertson et al. 1999), or even lacking among sympatric conspecific morphs (Seehausen et al. 1999b). In addition, the promiscuous mating system of these cichlids, with exclusive female parental care in the form of mouthbrooding, is conducive to strong sexual selection by female mate choice.

Although the cichlid fish in Lake Victoria show many characteristics that are consistent with sympatric speciation by sexual selection on male nuptial coloration, recent theoretical models indicate that sympatric speciation by sexual selection alone (Arnegard & Kondrashov 2004; Kirkpatrick & Nuïsmer 2004; Van Doorn et al. 2004), and even sympatric speciation much more generally, requires stringent conditions (Gavrilets 2004). In the absence of ecological differentiation, it is unclear how novel phenotypes can invade. Also, the stable co-existence of incipient species is problematic, both during and after speciation. All this is due to a lack of frequency-dependent selection (Van Doorn et al. 2004). Van Doorn et al. (2004) and Lande et al. (2001) showed that frequency-dependent selection can be generated by female-female competition for mates. However, even under these conditions sympatric speciation was only observed when additional and independent sources of disruptive selection were assumed. Van Doorn et al. (2004), Seehausen & Schluter (2004) and Mikami et al. (2004) independently proposed...
that male-male competition for access to breeding territories could generate such disruptive selection. Males of Lake Victoria and Malawi cichlid fish establish breeding territories to attract females and defend these territories aggressively against other males. Usually, only territorial males participate in reproduction (Parker & Kornfield 1996; Maan et al. 2004). Hence, competition over territory sites is intense, and is likely to exert sexual selection (Fryer & Isles 1972; Seehausen et al. 1998b). Simulation models (Van Doorn et al. 2004) indicate that if territorial individuals direct more aggression to phenotypically similar than to dissimilar rivals, sympatric speciation by sexual selection is theoretically feasible. Such a bias in aggression to like-colored males would result in a fitness advantage of males of a rare phenotype relative to males of the more abundant phenotype in a given local cichlid community. This advantage is negatively frequency-dependent, because as the frequency of the rare phenotype increases, its advantage decreases due to more aggressive encounters with its own type. This would facilitate the invasion of both types, but would prevent either type to drive the other extinct. In this way male-male competition can support co-existence of several morphs and, hence, stabilize the speciation process.

Traditionally, intra- and interspecific aggression has been studied to understand co-existence through ecological resource partitioning (e.g. Ebersole 1985; Ribbink 1991; Genner 1999b). Experimental evidence exists that some fish direct more aggression towards conspecifics than towards heterospecifics (Losey 1981; Itzkowitz 1990; Kohda 1998), although other studies are ambiguous (Brockmann 1973; Thresher 1978; Draud & Itzkowitz 1995; Genner 1999b; Leiser 2003). Genner and coworkers (1999b) showed that territorial male cichlids of the *Pseudotropheus* species complex in Lake Malawi never tolerated males and females of the same species complex in their territories, with a stronger aggression bias for dietary specialists than for dietary generalists. Further, Lake Malawi cichlids have non-overlapping territories at the intrageneric level, but males that belong to different genera may have overlapping territories (McKaye 1991). Fighting heterospecifics is likely to be a waste of energy and time, unless they are direct competitors for ecological resources. Between ecologically similar incipient and sibling species, the same might be true for competition for mates. However, studies that focus on differential aggression among ecologically similar species are lacking, and the possible role of intra- and interspecific aggression in sexual selection and speciation has largely been neglected (Seehausen & Schluter 2004).

In laboratory and field experiments, we tested for aggression biases in two sympatric pairs of red and blue sibling species of *Pundamilia* that are similar in ecology and anatomy. Sympatric red/blue or yellow/blue sibling species pairs are common in haplochromine cichlid communities (Seehausen & Van Alphen 1999; Seehausen & Schluter 2004). In a laboratory experiment, we tested a pair of fully sympatric partially reproductively isolated species by presenting territorial males of either color type with stimulus males of both types. To test for the effect of nuptial coloration on biasing aggression, the experiment was carried out once under white
light and once under green light that effectively masked the color differences between red and blue males. In a field experiment, we tested a pair of an apparently reproductively isolated sibling species in the field under natural light conditions. For both experiments, we predicted that males of each phenotype direct more aggression towards males of their own color type.

**Methods**

*Experiment A: partially reproductively isolated red and blue species pair*

**Species and subjects**

The pair of occasionally hybridizing species consisted of a *Pundamilia nyererei* (Witte-Maas & Witte 1985)-like and a *Pundamilia Pundamilia* (Seehausen et al. 1998a)-like phenotype, both from Python and Kissenda Islands in the Mwanza Gulf of Lake Victoria (Tanzania). Males of *P. nyererei*-type are crimson dorsally, yellow on their flanks, and have a crimson dorsal fin. We refer to this red phenotype as ‘red morph’. Males of *P. Pundamilia*-type are greyish white dorsally and on the flanks, and have a metallic blue dorsal fin. We refer to this phenotype as ‘blue morph’. Both are confined to rocky shores and islands in Lake Victoria (Seehausen 1996), and occur syntopically around Python and Kissenda Islands. Differentiated red and blue phenotypes are common around Python and Kissenda Islands, with intermediate forms occurring at lower frequencies (Seehausen 1997 unpublished; chapter 3). Males were brought from the Mwanza Gulf into the Zoological Laboratory in Haren in May 2001. We used only wild-caught males, 26 red and 23 blue males.

**Housing**

Males and females were kept in stock aquaria of 100 cm x 45 cm x 38 cm with 13-25 individuals of the same morph per aquarium. Experimental aquaria were of the same size. The short sides and the back of the aquaria were covered with black plastic. All aquaria were connected to a central biological filter system and water circulated continuously. Water temperature was kept at 25 ± 2°C and a 12:12 h light:dark cycle was maintained. The bottom of aquaria was covered with gravel. Fish were fed flake food (TetraMin Tropical Fish Flakes) six days of the week.

Prior to the experiments, males were individually housed for at least two months in separate compartments within which a PVC tube was placed as a refuge. Males had visual contact with one male of the same color through a transparent plastic sheet. This arrangement ensured that males became territorial (illustrated by aggressive displays and butting behavior towards the neighboring male) and avoided unwanted effects of social isolation, while preventing them to engage in physical interaction.
**Aggression choice tests**

An experimental tank consisted of a central compartment (60 cm x 45 cm x 38 cm) for the test fish. On both sides of this central compartment, we created two side compartments (20 cm x 45 cm x 38 cm) for dither fish, using transparent partitions. Each side compartment contained three to four juvenile fish with no nuptial coloration (*P. Pundamilia* from Kissenda Island, standard length (SL) 2-2.5 cm). Visual exposure to these dither fish ensured that the test fish retained territorial condition. A test male was placed in the central compartment one day before a trial for acclimatization. A PVC tube was provided as a refuge. At the start of a trial, two stimulus males, one red and one blue male (see below), were confined in transparent watertight tubes and placed at the right and the left ends of the central compartment. The behavior of the test male was recorded on videotape for 10 minutes from behind a blind. The stimulus males were switched between the two sides halfway through the test to correct for any possible side biases. Each stimulus pair was used for one blue and one red test male to allow for pairwise comparison of blue and red male behavior. Across trials we alternated the initial left and right position of red and blue stimulus males.

Standard length (SL) of each male was measured to the nearest 0.1 mm with calipers. Stimulus pairs were formed by matching a blue and a red male for SL. The size asymmetry, calculated as (L-S)100/S, where L is SL of the larger, S that of the smaller fish, was less than 7%. Red and blue males did not differ in average SL within pairs (Wilcoxon Signed Rank tests (WSR), Z=-1.601, P=0.109, N=15). Due to a limited stock of wild-caught fish, most males acted both as test and stimulus male in different trials. The interval between re-using fish was at least five days.

We tested 15 blue and 15 red males: blue test males were on average larger than red test males (blue males N=15, median=94.8 mm, range=85.8-118.7, red males N=14 [one omitted due to courtship, see below], median=89.1, range=81.4-106.8, WSR, Z=-2.198, P=0.028, N=14) which reflects the situation at Python and Kissenda Island where blue males tend to attain a larger body size than red males (Seehausen 1996).

**Light manipulation**

To isolate effects of nuptial coloration of stimulus males from possible effects of other differences between them on aggression bias in the test male, we did each test once under white and once under green light. Illumination was provided by a 58 W fluorescent light tube (Osram L58W/21-840 cool white) in hoods suspended 80 cm above the water surface. Green light was created with a single layer of green sheets (Filter Primary Green, code 139, Lee Filters). To have white and green light of similar intensities, we wrapped two layers of neutral filters (Filter Neutral Density, code 209 0.3ND, Lee Filters) around the hoods in the white light treatment which decreases light intensity without loss of spectrum. Light intensity was measured at 8 fixed points in the experimental room. White light was 13% more intense than
green light (White light: X ± SD = 224.25 ± 19.83 lux, N=8, green light: X ± SD = 252.75 ± 26.14, N=8). Under both light treatments males exhibited the full range of aggressive behaviors. After 5-8 trials in one light treatment, corresponding to one day, we replaced the filters (green light or white light, see below). The next day we repeated the test of the males of the previous day under the second light treatment with the same set of stimulus males. Sets of trials were balanced and alternated with respect to the sequence of the light treatment.

Behavioral observations

The videotapes were analyzed with a Video Interface and Time Code Generator using Observer Software version 3.0 (Noldus Information Technology, Wageningen, The Netherlands). The following behavior patterns were recorded (see Baerends & Baerends-van Roon 1950): display behavior (i.e. frontal and lateral display) and attack behavior (i.e. biting and butting) at the walls of the tubes that contained the stimulus males. A display event was defined as lateral or frontal display, ending with a change in posture. An attack bout was defined as a series of rapid bite attempts with <1 second time intervals between the bites. The Observer Software yields both time budgets and frequencies of behaviors.

Experiment B: fully reproductively isolated red and blue species pair

Species and subjects

The fully reproductively isolated species pair consisted of *Pundamilia nyererei* (Witte-Maas & Witte 1985) and *Pundamilia 'pink anal'* (an as yet undescribed species, Seehausen 1996) from Makobe Island in the Speke Gulf of Lake Victoria (Tanzania). Hybrid phenotypes have never been seen at Makobe Island (Seehausen et al. 1998b; Dijkstra, Maan and Seehausen personal observation). *P. nyererei* from Makobe Island is referred to as ‘red species’. It differs from the *P. nyererei*-like individuals from Python and Kissenda Islands in that males are brighter red. Males of *P. 'pink anal'* are dark metallic blue and have a characteristic pink anal fin and pink lappets on the dorsal fin. We refer to them as ‘blue species’. Both species occur sympatrically and syntopically around Makobe Island (for details see Seehausen 1996; Seehausen & Bouton 1997). Their breeding sites fully overlap, but *P. 'pink anal'* is less abundant than *P. nyererei* on the breeding site (Seehausen et al. 1998b). The relatively clear waters make Makobe Island one of the few locations in Lake Victoria where experimental work using SCUBA is possible. We used 27 red and 25 blue males.

Aggression choice tests

Fieldwork was conducted by SCUBA diving. Territorial males of both species were located along a transect line at a depth of six to nine meters. Each male territory
was marked with a coded tile placed between the rocks. A diver positioned a red and a blue stimulus male (size-matched, standard length asymmetry <6%), each individually confined in a watertight transparent tube, into the centre of a territory. Stimulus males were obtained by gillnetting or angling at nearby locations around Makobe Island. The centre of a male’s territory was defined as the most frequent position of the territory owner, usually at a pile of rocks or close to a crevice. Typically, upon placing a stimulus pair, the territorial male either retreated in a crevice or fled, and reappeared within a minute. If a male did not return or respond within the first five minutes the trial was aborted. We successfully tested 14 blue and 12 red males.

A diver recorded the frequencies of display events and attack bouts to each stimulus male with a pencil on a PVC sheet. Like the laboratory tests, each field trial lasted 10 minutes, and halfway through the trial we switched the left right positions of the stimulus males.

Analysis

Experiment A

In experiment A, under white light one blue male, and under green light two red males and three blue males performed courtship behavior. These cases were excluded from the analysis. Since neither response ratios, nor aggression level differed between males from Python and Kissenda Islands (Mann-Whitney U tests (MWU), response ratios, P>0.1, aggression level, P>0.1), we pooled the data. To analyze whether a bias exists in aggression, we first generated response ratios based on frequencies of, as well as time spent on both display and attack behavior. The display duration ratio of each male was expressed as the time spent displaying towards a red stimulus (D_r) relative to the total time spent displaying towards both stimuli, calculated as: \[ D_r/(D_r+D_b) \]. We calculated the display frequency ratio in the same way by using frequencies of displays. The attack duration and frequency ratios were generated in an analogous way. The aggression level was defined as the sum of frequencies of and the time spent displaying and attacking to both the red and blue stimulus males.

To test whether males had an aggression bias towards either their own or the other morph, the attack and display ratios were tested against 50% using Wilcoxon matched-pairs signed-rank (WSR) tests. We used WSR tests also to compare the response ratios between morphs tested with the same stimulus pair and between light treatments using the same test pairs. All tests were two-tailed, except for the light treatment comparison of red males, where we were able to make an a priori prediction about the direction of the results, namely that masking color differences would only lead to less discrimination and not to more discrimination of red and blue stimuli. Sample sizes varied depending on the type of comparison or test. For example, if one male performed courtship behavior
under green light, the corresponding case under white light was omitted to allow for pairwise comparison. Differences in overall aggression levels between morphs and light treatments were also compared with WSR tests.

**Experiment B**

Experiment B was analyzed like experiment A. However, we calculated response ratios and aggression level only from frequencies of display events and attack bouts. In addition, differences between species were examined with MWU tests, because stimulus pairs could not always be presented pairwise to one male of each species. Each stimulus pair was only presented to a single male of each species, except for four stimulus pairs which were each presented to two different blue males. For the analysis of aggression level we treated these data point as independent events (blue males, N=14). However, for the analysis of response ratios, we only took those cases with the highest aggression level to avoid pseudoreplication (blue males, N=10), although the analysis including all cases yielded the same P-values.

**Comparison between experiment A and B**

The response frequency ratios between red and blue morph from the Mwanza Gulf and red and blue species from Makobe Island were compared with MWU tests.

Table 1 Experiment A: tests against 50% of response ratios (figure 1A, 1B) of red (white light N=14, green light N=12) and blue males (white light N=15, green light N=12) in a partially reproductively isolated species pair. Z-values and significance are indicated for WSR-tests. All tests are two-tailed.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Red morph</th>
<th>Blue morph</th>
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<tr>
<td></td>
<td>White light</td>
<td>Green light</td>
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<tr>
<td></td>
<td>Z</td>
<td>P-value</td>
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<tr>
<td>Display ratio</td>
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<td>Duration</td>
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<tr>
<td>Attack ratio</td>
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<td>Duration</td>
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</tr>
</tbody>
</table>

**Results**

**Experiment A: partially reproductively isolated red and blue species pair**

**White light**

Red males paid on average slightly more attention to red than to blue stimuli: the display frequency ratio tended to deviate from 50% (table 1, figure 1A), and the
display duration ratio deviated significantly from 50% (table 1, figure 1B). Both the attack frequency and duration ratios tended to deviate from 50% (table 1, figure 1A, 1B). In contrast, blue males did not discriminate between the two stimuli: none of the response ratios deviated from 50% (table 1, figure 1A, 1B). Response ratios of blue and red males did not differ significantly (WSR tests: -1.412<Z<-0.282, 0.158<P<0.778, N=14). When the ratios of blue and red males were pooled, the response ratios deviated from 50%, although this was only significant for the duration ratios: (WSR-tests: display frequency ratio: Z=-1.435, P=0.151, display duration ratio: Z=-2.152, P=0.031, attack frequency ratio: Z=-1.676, P=0.094, attack duration ratio: Z=-2.303, P<0.021, N=29). This indicates that overall, red males evoked more aggression than blue males.

Figure 1A and 1B The display (grey) and attack ratios (open) of red and blue males in the partially reproductively isolated species pair, based on frequencies (figure 1A) and durations (figure 1B). Response ratios are presented for white light (red males N=14, blue males N=15) and green light condition (males of both morphs N=12). The response ratio is the response to the red stimulus divided by the sum of the responses to the red and blue stimuli. A response ratio of 50% represents identical responses to the two stimuli (----). Geometric means are presented with the 25th and the 75th percentiles and error bars show the 10th and 90th percentiles.
Light treatment effect

Under green light, neither red nor blue males discriminated between red and blue stimuli (table 1, figure 1A, 1B). For red males, all response ratios differed between the two light treatments (table 2, Figure 1A, 1B). Such a difference was not observed for blue males (table 2, figure 1A, 1B). These results indicate that the bias of red males towards aggressing red competitors more than blue ones was due to the differences in nuptial coloration between the stimulus males, rather than other differences between the morphs.

Under white light red males spent significantly more time displaying than blue males (table 3). Under green light there was no difference in aggression level between morphs (table 3). For red males, attack duration was shorter under green light than under white light, but the other measures of aggression level did not differ between light treatments (WSR-tests: display frequency: $Z=-0.157$, $P=0.875$, display duration: $Z=-0.157$, $P=0.875$, attack frequency: $Z=-0.039$, $P=0.969$, attack duration: $Z=-2.197$, $P=0.028$, $N=12$). The aggression level of blue males did not differ between light treatments (WSR-tests: display frequency: $Z=-0.039$, $P=0.969$, display duration: $Z=-1.177$, $P=0.239$, attack frequency: $Z=-0.667$, $P=0.505$, attack duration: $Z=-0.157$, $P=0.875$, $N=12$).

Experiment B: fully reproductively isolated red and blue species pair

Both red and blue males directed more display behavior to males of their own species (figure 2, WSR-tests: red: $Z=-2.578$, $P=0.010$, $N=12$, blue: $Z=-2.073$, $P=0.038$, $N=10$). The attack ratio deviated from 50% only for red males (WSR-tests:

![Graph showing the display and attack ratios of blue and red males.](image)

**Figure 2** The display (grey) and attack ratios (open) of blue males ($N=14$, statistics based on $N=10$) and red males ($N=12$) of a sympatric and fully reproductively isolated species pair based on frequencies. The response ratio is the response to the red stimulus divided by the sum of the responses to the red and blue stimuli. A response ratio of 50% represents identical responses to the two stimuli (------). Geometric means are presented with the 25th and the 75th percentiles and error bars show the 10th and 90th percentiles.
red: Z=-2.835, P=0.005, blue: Z=-0.415, P=0.678). Both display and attack ratios to the red versus the blue species differed significantly between the species (display ratio: MWU=9.5, P<0.001, N₁=12, N₂=10, attack ratio: MWU=21.5, P=0.009). Red males exhibited a stronger bias in attack to conspecifics than blue males (MWU=18.5, P=0.004, N₁=12, N₂=10), but the strength of the display bias to conspecifics did not differ between the species (MWU=46.5, P=0.381). Overall, blue and red males directed more displays (Z=-3.323, P=0.001, N=22) and attacks (Z=-2.542, P=0.011, N=22) to their own species. Blue and red males did not differ in the absolute frequency of attacks and displays (table 4).

Comparison between partially and fully reproductively isolated species pair

The own species bias in aggression exhibited by the fully reproductively isolated red species tended to be stronger than the own morph bias by the red morph of the partially reproductively isolated species, significantly so for the attack frequency ratio (display frequency ratio: MWU=55, P=0.145, attack frequency ratio: MWU=33, P=0.008, N₁=12, N₂=14). The display bias of the blue species towards conspecifics was significantly stronger than the bias in the blue morph (display frequency ratio: MWU=31.5, P=0.014, N₁=10, N₂=15), but this difference was not observed in the attack frequency ratio (MWU=59, P=0.397).

Table 2 Experiment A: comparison of response ratios (figure 1A, 1B) between white and green light of red and blue males (males of both morphs N=12) in a partially reproductively isolated species pair. Z-values and significance are indicated for WSR-tests. Tests for red males are one-tailed, tests for blue males two-tailed.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Red morph</th>
<th></th>
<th></th>
<th>Blue morph</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z</td>
<td>P-value</td>
<td>Z</td>
<td>P-value</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Display ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency</td>
<td>-1.804</td>
<td>0.036</td>
<td>-0.157</td>
<td>0.875</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration</td>
<td>-1.804</td>
<td>0.036</td>
<td>-0.941</td>
<td>0.347</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Attack ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency</td>
<td>2.197</td>
<td>0.014</td>
<td>-0.628</td>
<td>0.530</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration</td>
<td>-1.647</td>
<td>0.0495</td>
<td>-1.098</td>
<td>0.272</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Discussion

It has recently been proposed that a bias in aggression towards the own species in male-male competition may facilitate stable co-existence of incipient species, both during and after speciation (Mikami et al. 2004; Van Doorn et al. 2004, Seehausen & Schluter 2004). The results in the present study are partly consistent with this hypothesis: males of reproductively isolated, but closely related red and blue
species that occur sympatrically and syntopically directed significantly more agonistic behavior towards conspecific males than towards males of the other species. In interspecies competition for territories, males of both species may, hence, experience elevated fitness when their species is locally the less abundant of the two. This mechanism may promote stable co-existence of red and blue phenotypes after speciation, even without ecological divergence.

Table 3 Experiment A: aggression level (median, 25th and 75th quartile) expressed as the sum of frequencies and time spent attacking and displaying towards both stimuli for red males (white light N=14, green light N=12) and blue males (white light N=15, green light N=12) of a partially reproductively isolated species pair under both light conditions. Z-values and significance is indicated for WSR-tests. Tests are two-tailed.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Red morph</th>
<th>Blue morph</th>
<th>Z</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (25th and 75th)</td>
<td>Median (25th and 75th)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Display</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency</td>
<td>64.50 (50.75-72.25)</td>
<td>46.00 (37.00-60.00)</td>
<td>-0.157</td>
<td>0.875</td>
</tr>
<tr>
<td>Duration</td>
<td>180.85 (142.43-189.61)</td>
<td>116.15 (96.83-152.30)</td>
<td>-2.103</td>
<td>0.035</td>
</tr>
<tr>
<td>Attack</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency</td>
<td>51.00 (38.00-60.25)</td>
<td>44.00 (30.00-55.00)</td>
<td>-0.039</td>
<td>0.969</td>
</tr>
<tr>
<td>Duration</td>
<td>231.70 (157.78-293.00)</td>
<td>190.50 (145.35-300.90)</td>
<td>-0.722</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Display</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency</td>
<td>57.00 (36.75-85.50)</td>
<td>47.50 (44.00-54.75)</td>
<td>-0.039</td>
<td>0.969</td>
</tr>
<tr>
<td>Duration</td>
<td>147.65 (90.28-195.10)</td>
<td>166.20 (103.45-197.00)</td>
<td>-0.51</td>
<td>0.6</td>
</tr>
<tr>
<td>Attack</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency</td>
<td>48.50 (34.50-57.25)</td>
<td>45.00 (37.25-54.25)</td>
<td>-0.667</td>
<td>0.505</td>
</tr>
<tr>
<td>Duration</td>
<td>190.60 (122.68-230.90)</td>
<td>211.55 (186.72-225.53)</td>
<td>-0.561</td>
<td>0.58</td>
</tr>
</tbody>
</table>

The aggression bias for conspecifics in reproductively isolated red and blue species is consistent with the findings of Seehausen & Schluter (2004). In a field survey on Lake Victoria cichlid communities, they found that closely related species that occur at the same locality tend to differ markedly in coloration. In the same study it was found that territorial males tend to have territorial neighbors of species that are different in nuptial coloration from themselves (Seehausen & Schluter 2004). There is evidence that a similar segregation pattern among territorial males of several co-existing species also exists in cichlid communities in Lake Tanganyika (Kohda 1998). These patterns can be explained when individuals
among closely related species bias their aggression towards conspecifics and males of phenotypically similar species, exerting negative frequency-dependent selection on male nuptial coloration (Seehausen & Schluter 2004).

Table 4 Experiment B: aggression level (median, 25th and 75th quartile) expressed as the sum of frequency of displays and attacks directed to both stimuli for red males (N=12) and blue males (N=14) of a fully reproductively isolated species pair. Significance is indicated for MWU-tests. Tests are two-tailed.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Red species</th>
<th></th>
<th>Blue species</th>
<th></th>
<th>MWU</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Display</td>
<td>48.50 (38.00-69.50)</td>
<td>39.00 (32.75-57.00)</td>
<td>69.0</td>
<td>0.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Attack</td>
<td>22.00 (7.25-45.50)</td>
<td>29.50 (12.50-45.75)</td>
<td>74.0</td>
<td>0.63</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

We expected own-type biases also in males of the partially reproductively isolated species pair. Inconsistent with this hypothesis, blue males in the partially reproductively isolated species pair that we studied did not exhibit any aggression bias to males of their own color type. Consistent with the hypothesis, however, red males in the same pair were more aggressive to other red males than to blue males. Although the analysis on the basis of behavioral frequencies did just not reach statistical significance, the analysis on the more accurate basis of time spent on aggression yielded significant results. Although these color effects on male-male aggression will cause the fitness of red phenotypes to be maximized when red is rare, and to decrease with increasing relative abundance, they would never generate a fitness advantage of red over blue. Therefore, it appears that in the case reported here, aggression bias alone does not stabilize the polymorphism. However, results of a previous investigation (chapter 4) suggest that the disadvantage that males of the red morph would suffer from the color-based aggression bias in the partially reproductively isolated species pair, may well be offset by a significant color-based dominance advantage of red males in physical combat in the same species pair (chapter 4). It is possible that the two together stabilize the polymorphism.

Importantly, the assumption that aggression bias is based on color differences and not on other morph-specific traits is indicated by our light manipulation experiment. Males of the red and blue morph did not distinguish between red and blue stimulus males under green light, and the aggression bias of red males under white light was significantly reduced under green light. It appears, therefore, that nuptial color in Pundamilia is important not only in female mate choice (Seehausen & Van Alphen 1998), but also in intrasexual signaling.

The adaptive significance of differential aggression in promoting the establishment of polymorphisms and co-existence of species can be understood by taking into account the degree of competition for females. Gene-flow between red
and blue in the partially reproductively isolated species pair suggests that males of the red and blue morph compete partly for the same set of females. This may render it less advantageous for blue males to concentrate aggression on males of own color than in a situation where red and blue are fully reproductively isolated species, e.g. at Makobe Island. This may explain the difference in own-type biases between the partially and the fully reproductively isolated species pair that we studied.

The own-type bias of red males was stronger than that of blue males in both the partially and the fully reproductively isolated species pair. The deviation from symmetry in frequency-dependent selection caused by male-male aggression biases suggests that blue *Pundamilia* phenotypes are likely to be favored by male-male competition at least unless blue is clearly more abundant than red. This may explain why red *Pundamilia* populations appear to always occur sympatrically with at least one blue species or color morph, whereas entirely blue populations are not uncommon (Seehausen & Van Alphen 1999).

Taken together, our results suggest that (1) red phenotypes have a stronger own-type bias than blue phenotypes; that (2) an own-type bias in aggression alone is not likely to promote co-existence of partially reproductively isolated species, and that (3) aggression bias may stabilize syntopic co-existence of reproductively isolated sister species.

**Acknowledgements**

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Male-male competition and speciation: aggression bias towards differently colored rivals varies between stages of speciation in a Lake Victoria cichlid species complex

Peter D. Dijkstra, Ole Seehausen, Michele E.R. Pierotti, Ton G. G. Groothuis

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Abstract

Sympatric speciation driven by sexual selection by female mate choice on a male trait is a much debated topic. The process is problematic due to the lack of negative frequency-dependent selection that can facilitate the invasion of a novel color phenotype and stabilize trait polymorphism. It has recently been proposed that male-male competition for mating territories can generate frequency-dependent selection on male coloration. Rare males would enjoy a fitness advantage if territorial defenders bias aggression towards males of their own color. We used blue (ancestral type) and red phenotypes of the Lake Victoria cichlid species complex *Pundamilia*. We tested the aggression bias of wild-caught territorial blue males from five separate populations for blue versus red rival males using simulated intruder choice tests. The different populations vary in the frequency of red males, and in the degree of reproductive isolation between red and blue, reflecting different stages of speciation. Blue males from a population that lack red phenotypes biased aggression to blue stimulus males. The same was found in two populations where blue and red are reproductively isolated sister species. This aggression bias may facilitate the invasion of a novel color phenotype and species co-existence. Blue males from two populations where red and blue are hybridizing incipient species biased aggression towards red stimulus males. Thus, after successful invasion of red, aggression bias alone is not likely to generate frequency-dependence required to stabilize the co-existence of phenotypes. The findings show that aggression bias varies between stages of speciation, but is not enough to stabilize the process of speciation.
Introduction

Empirical research on potential mechanisms of sympatric speciation has concentrated primarily on disruptive natural selection through competition for ecological resources leading to the evolution of ecological specialization and reproductive isolation (Filchak et al. 2000; Schluter 2000; Turelli et al. 2001; Rundle & Nosil 2005). This partly reflects the developments in theoretical research: in classical theoretical scenarios of sympatric speciation, sexual selection plays a secondary role if any (Maynard Smith 1966; Bush 1975; Rosenzweig 1978; Kondrashov & Mina 1986; for review see Coyne & Orr 2004). Different speciation models have demonstrated the theoretical feasibility that selective mating exerting sexual selection can cause sympatric speciation. (Lande & Kirkpatrick 1986; Turner & Burrows 1995; Payne & Krakauer 1997; van Doorn et al. 1998; Higashi et al. 1999; Kawata & Yoshimura 2000; Van Doorn & Weissing 2001). Many of these models were inspired by the explosive speciation of haplochromine cichlids in Lake Victoria and Lake Malawi (Kocher 2004). Closely related haplochromine cichlid species typically differ markedly in coloration, but little else (Bouton et al. 1997; Seehausen & Bouton 1997; Albertson et al. 1999; Genner et al. 1999a; Allender et al. 2003). This color variation in males is associated with variation in female mate preferences (Seehausen & Van Alphen 1998; Knight & Turner 2004).

More recent theoretical investigations indicate that sympatric speciation by sexual selection alone requires stringent conditions (Turelli et al. 2001; Arnegard & Kondrashov 2004; Kirkpatrick & Nuismer 2004; Van Doorn et al. 2004). A fundamental problem is the initial hurdle for a novel male phenotype to invade a population against the predominant female preference. Also, the co-existence of incipient species, both during and after the evolution of reproductive barriers, is problematic (Van Doorn et al. 2004). Successful invasion requires a fitness advantage for a novel male phenotype, but maintenance of a trait polymorphism requires that this advantage is negatively frequency-dependent (Van Doorn et al. 2004). Male-male competition for access to mating territories could generate such frequency-dependence in sexual selection when exerting selection on the same traits that are involved in female mate choice (Mikami et al. 2004; Seehausen & Schluter 2004; Van Doorn et al. 2004). For example, if territorial defenders bias aggression towards phenotypes colored like themselves, rare male phenotypes would receive less aggression, possibly resulting in a higher chance of rare male types to establish a high-quality territory. If females assess prospective mates not only by color but also territory quality (for example territory size, see Maan et al. 2004; chapter 7), they may face a conflict between species recognition and preference for mates with a high-quality territory. This effect could bestow an initial fitness advantage upon rare males facilitating the propagation of their genes in the population. The advantage is likely to decrease with increasing relative abundance due to more aggressive encounters with own-type individuals. This ‘rare male advantage’ would facilitate both the invasion of novel color types, and prevent
competitive exclusion of either type. It could potentially operate in the haplochromine cichlid radiations. In their lek-like mating system, males vigorously defend permanent territories, do not take part in parental care, and it appears that possession of a territory is a prerequisite for mating success (Maan et al. 2004). Hence, competition over territory sites is intense, and may well exert sexual selection (Fryer & Isles 1972; Seehausen et al. 1998b; Seehausen & Schluter 2004).

Intra- and interspecific aggression have mostly been studied to understand coexistence through ecological resource partitioning (e.g. Ebersole 1985; Martin & Thibault 1996; Genner et al. 1999b; for reviews in haplochromines see McKaye 1991; Genner & Turner 2005). Genner et al. (1999b) showed that in the haplochromine cichlid *Pseudotropheus* from Lake Malawia, territorial behavior might promote coexistence of species with different feeding strategies, while enhancing competition among species with a similar specialization. The role of aggression in sexual selection and speciation has only recently been fully appreciated (Mikami et al. 2004; Seehausen & Schluter 2004; Van Doorn et al. 2004; this thesis).

Here we tested for aggression biases in wild-caught territorial blue male phenotypes of the *Pundamilia* species complex from Lake Victoria towards either red (*P. nyererei*) or blue (*P. pundamila*) rivals using simulated intruder choice tests. Red and blue phenotypes are anatomically similar that behave like reproductively isolated species in some locations, and like hybridizing incipient species in other locations (Seehausen 1996; Seehausen 1997). Blue phenotypes have a lake-wide distribution whereas red phenotypes have a patchy distribution and always co-occur with blue phenotypes. Among the members of the *Pundamilia* complex, the blue form has the highest record of sympatric occurrences with other members of the *Pundamilia* complex (Seehausen 1996; Seehausen & Van Alphen 1999). It seems therefore likely that blue represents the ancestral state and that blue populations have been invaded repeatedly and independently by red phenotypes during speciation (Seehausen 1997; Seehausen & Van Alphen 1999; Seehausen & Schluter 2004).

In this study we have taken advantage of the fact that blue *Pundamilia* populations exist that differ in the frequency of red males, as well as in the degree of gene-flow between red and blue phenotypes (Seehausen 1997). We take these population types as representing different stages of speciation, allowing us to ask whether the direction and strength of an aggression bias in blue males depends on speciation stage. Firstly, we measured aggression bias in wild-caught males from a location with a single blue *Pundamilia* species (single-species population). This population represents the pre-invasion stage. Secondly, we measured the aggression bias in wild-caught blue males from two populations where blue and red are clearly distinguishable incipient species or color morphs, in which intermediate (hybrid) phenotypes are present (hybridizing-species populations). This population type represents an incipient stage of speciation after invasion and increase in frequency of red males. Finally, we measured the aggression preference in wild-
caught blue males from two populations where blue and red are completely distinct species (two-species populations). This population type represents a situation where reproductive isolation has been completed. If male-male competition is to facilitate speciation, we predict own-type biases in aggression in all population types (Mikami et al. 2004; Seehausen & Schluter 2004; Van Doorn et al. 2004). However, a previous study on *Pundamilia* indicates that own-type-aggression biases do not always exist. In this study we found that red males from different populations biased aggression towards males of their own color (chapter 2). In contrast, in the same study blue males from hybridizing-species populations showed a nonsignificant bias towards red intruders (chapter 2). This suggests that theory does not completely account for what actually happens in nature and that aggression biases of blue males may depend on speciation stage. This warrants sampling of aggression biases in more blue populations that differ in the degree of speciation, including a population consisting of a single blue species.

**Method**

**Species and subjects**

*Pundamilia Pundamilia* (Seehausen et al. 1998a) and *Pundamilia nyererei* (Witte-Maas & Witte 1985) are endemic Lake Victoria cichlid species. Males were brought from the Mwanza Gulf into the Zoological Laboratory in Haren in February 2003. Some males from Python and Kissenda Island arrived in May 2001. We only tested wild-caught males. Males of *P. nyererei* are crimson dorsally, yellow on their flanks, and have a crimson dorsal fin. We refer to these as ‘red’. Males of *P. Pundamilia* are greyish white dorsally and on the flanks, and have a metallic blue dorsal fin. We refer to them as ‘blue’. Both are confined to rocky shores and islands in Lake Victoria (Seehausen 1996). Below we describe the five populations from which we collected blue males. The frequencies of color phenotypes in the wild for each population are shown in table 1.

The *single-species population* originated from Luanso Island. This population consists of individuals that show a continuous distribution of colors between blue and red (Seehausen 1996; 1997). Most Luanso *Pundamilia* males are similar to *Pundamilia Pundamilia*, hence we refer to these males as ‘blue’. Some other males are intermediate between the blue and the red phenotype, but distinctly red males (score 4 and upwards) are extremely rare, and fully red males (score 5) have not been reported (see table 1).

The *hybridizing-species populations* consisted of *P. nyererei*- and *P. Pundamilia*-like phenotypes from Python and Kissenda Islands, in the Mwanza Gulf of Lake Victoria (Tanzania). Around these islands, red and blue occur syntopically. Differentiated red and blue *Pundamilia* are common around these islands, together with intermediate forms that are less abundant and closely
resemble laboratory hybrids (table 1). We only used distinct blue and red male phenotypes (males with color score 0 and 5 respectively).

The two-species populations consisted of fish from Makobe Island and Senga Point, in the Speke Gulf of Lake Victoria (Tanzania). At these locations *Pundamilia Pundamilia* and *Pundamilia nyererei* are reproductively isolated sympatric sibling species since intermediate phenotypes have never been observed among hundreds of fishes (table 1). At Senga Point they are also fully syntopic, whereas depth ranges do not overlap much at Makobe Island.

In this experiment we tested for aggression bias in wild-caught blue males towards either red or blue stimulus males. We tested 28 males from Luanso Island (standard length, [SL] mean ± SE=92.8 ± 1.3mm), 22 males from Python Island (SL mean ± SE=96.6 ± 2.1), 7 from Kissenda Island (SL mean ± SE=98.4 ± 3.4), 23 *P. Pundamilia* males from Makobe Island (SL mean ± SE=114.0 ± 2.5), and 11 males from Senga point (SL mean ± SE=115.24 ± 2.8).

Stimulus males were blue and red males from Kissenda or Python Island, scored either as 0 or as 5 phenotypes respectively. Kissenda and Python males are indistinguishable. In light manipulation experiments in which we masked differences in nuptial coloration of red and blue stimulus males from these islands, we have shown that aggression biases are based on differences in nuptial coloration rather than other possible morph-specific differences (chapter 2). This makes these males suitable stimuli to measure color-based aggression preferences. Males of all five populations were tested with stimulus males bred from wild-caught parents from Kissenda Island. Some males from Makobe Island (10 out of 23) were also tested with wild-caught stimulus males from Kissenda Island. Specifically, we selected red stimulus males from a group of 58 red *Pundamilia* males (SL range: 68.5-103 mm, 40 lab-bred and 2 wild-caught Kissenda males, 6 lab-bred and 10 wild-caught Python males), and blue males from a group of 50 blue *Pundamilia* males (SL range: 66.3-105, 35 lab-bred Kissenda males, 7 lab-bred and 8 wild-caught Python males). Any stimulus male was only used once for each test population. The size asymmetry within stimulus pairs, calculated as \((L-S)100/S\), where \(L\) is SL of the larger, \(S\) that of the smaller fish, was less than 6.2%.

<table>
<thead>
<tr>
<th>Phenotype score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luanso Island</td>
<td>6</td>
<td>16</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Python Island</td>
<td>19</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>Kissenda Island</td>
<td>8</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Makobe Island</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>55</td>
</tr>
<tr>
<td>Senga Point</td>
<td>27</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 1 The frequency of *Pundamilia* color phenotypes (0-5: 0 = pure blue and 5 = pure red, for hybrid color scale see online publication in Journal of Evolutionary Biology) shown for each population. The fish were collected by hook and line and gillnets. The data of Python and Kissenda Island are from 2001, that of the other populations from 2002/2003.
**Housing**

The test males were kept in single-population aquaria with wild-caught females and only blue males. Males from different populations were housed in separate aquaria. The sides and the back of the aquaria were covered with black plastic sheets. All aquaria were connected to a central biological filter system and water circulated continuously. Water temperature was kept at 25 ± 2°C and a 12:12 h light:dark cycle was maintained. All aquaria contained a gravel substrate. The fish were fed flake food (TetraMin Tropical Fish Flakes) seven times per week and a mixture of ground shrimp and peas two times per week.

Test and stimulus males were transferred to individual aquarium compartments at least a week prior to commencement of the simulated intruder choice tests in order to minimize the effects of short-term experiences. All males were kept in separate compartments within which a PVC tube was placed as a refuge. They had visual contact to a male through a transparent plastic sheet. Test males were visually exposed to only blue males, stimulus males to both red and blue males. This pre-experimentation housing in aquarium compartments ensured that males became territorial and avoided unwanted effects of social isolation, while preventing physical interaction.

**Simulated intruder choice test**

Test males were placed in the test aquarium one to three days before a trial to allow acclimatization to the aquarium. A PVC tube was provided as a refuge. A test aquarium consisted of a large experimental compartment (55 x 35 x 39 cm high) for the test male, and a smaller compartment (5 x 35 x 39 cm high) for a neighboring male. The neighboring male was a small lab-bred *P. Pundamilia* male (Kissenda or Python Island), which was required to maintain territorial condition of the test male. The neighboring male was separated from the test male by a transparent partition. At the start of a trial, a blue and a red stimulus male confined in transparent watertight tube were simultaneously placed in the experimental compartment 20 cm apart from one another. The test males perceived these stimulus males as intruders, and typically responded aggressively to both stimulus males, whilst the neighboring fish was ignored. The trial was recorded on videotape for 5 minutes starting immediately after introduction of the stimulus males. Across trials we alternated the left and right position of the stimulus males with respect to color.

**Analysis**

From the videotapes we scored the number of attacks and display behaviors of the test male towards both stimulus males (Baerends & Baerends-Van Roon 1950). The display ratio of each male was the fraction of displays directed towards the blue stimulus calculated as the number of displays towards blue divided by the sum of displays towards blue and red. The attack ratio was generated in the same way. All
ratio data were arcsine square root transformed to meet assumptions of parametric testing. We tested against 50% using one-sample t-tests to test whether males have an aggression preference for either color type. To test for differences in aggression bias between populations we used Analysis of Variance (ANOVA) and Tukey HSD post-hoc tests. Statistical analyses were performed in SPSS 12.0.1. All quoted probabilities are for two-tailed tests of significance.

**Results**

Males directed aggressive behaviors to both types of stimulus males. On average, males performed 38.0 displays (± SE 2.7, N=91) and attacks 32.8 (± SE 2.3) during a five minute trial.

![Figure 1](image_url)

Figure 1 The display (grey) and attack ratios (open) of *Pundamilia* males from five populations. The different populations of *Pundamilia* phenotypes are indicated below with numbers: the single-species population (1: *Pundamilia*, Luanso Island, N=28), the hybridizing-species populations (2: *P. Pundamilia*-like phenotypes, Python Island N=22, 3: Kissenda Islands N=7) and the two-species populations (4: *P. Pundamilia* Makobe Island N=23, 5: *P. Pundamilia* Senga Point, N=11). The response ratio is the response to the blue stimulus divided by the sum of the responses to the red and blue stimulus. A response ratio of 50% represents identical responses to the two stimuli (-----). Data points indicate mean ± SE values. In most instances the response ratios deviated significantly from 50% (for statistics see text); significance of deviation from 50% is indicated by asterices (*P<0.05, **P<0.01, ***P<0.001).
Figure 1 shows the response ratios of blue males of each population. Both the display and the attack ratio differed significantly across the five populations (display: ANOVA: F_{4,86} =10.462, P<0.001, attack: F_{4,86}=12.498, P<0.001; Figure 1). Males from the single-species population directed significantly more aggression to blue than to red stimulus males (Figure 1; one-sample t-test: display: t_{27}=-3.176, P<0.005, attack: t_{27}=-4.883, P<0.001). In contrast, blue males from both hybridizing-species populations paid on average more attention to red than to blue stimulus males (Figure 1; one-sample t-tests: Python Island, display, t_{21}=3.073, P<0.01, attack: t_{21}=3.846, P<0.001; Kissenda Island, display: t_{6}=2.858, P<0.05, attack: t_{6}=3.247, P<0.05). Blue males from the two two-species populations biased their aggression significantly towards the blue stimulus male (Figure 1; one-sample t-tests: Makobe Island, display: t_{22}=-2.333, P<0.05, attack: t_{22}=-1.996, P=0.058; Senga Point, display: t_{10}=-4.242, P<0.005, attack: t_{10}=-2.593, P<0.05).

We can now test whether response ratios depend on stage of speciation. Within population type, or speciation stage, we found no differences in response ratios between populations (Kissenda versus Python Island, Tukey HSD: display: P=1.0, attack: P=0.97; Makobe Island versus Senga Point: display: P=0.48, attack: P=1.0). The response ratios in the single-species population differed significantly from those in the two hybridizing-species populations (single-species population versus Kissenda Island: Tukey HSD: display: P=0.14, attack: P<0.001; single-species population versus Python Island: display: P<0.01, attack P<0.001). The response ratios in the hybridizing-species populations differed from those in the two-species populations (Kissenda versus Makobe Island: Tukey HSD: display: P<0.05, attack: P<0.01; Kissenda Island versus Senga Point: display: P<0.001, attack P<0.01; Python Island versus Makobe Island: display: P<0.001, attack: P<0.001; Python Island versus Senga Point: display: P<0.001, attack: P<0.01). The response ratios in the single-species population did not differ from those in the two-species populations (single-species population versus Makobe Island: Tukey HSD: display: P=0.86, attack: P=0.96; single-species population versus Senga Point: display: P=0.08, attack: P=1.0).

**Discussion**

It has recently been proposed that male-male competition for mating territories may facilitate the invasion of novel male phenotypes and stabilize sympatric speciation and species coexistence through negative frequency-dependent selection (Mikami et al. 2004; Seehausen & Schluter 2004; Van Doorn et al. 2004). Such frequency-dependency may arise when territory owners bias aggression towards same-colored phenotypes (Mikami et al. 2004; Seehausen & Schluter 2004; Van Doorn et al. 2004). Blue *Pundamilia* males from the single-species population and the two two-species populations showed an aggression bias to blue stimulus males, consistent with the hypothesis. In contrast, blue males from the two hybridizing-species populations biased aggression towards red stimulus males. The findings are consistent with our expectation that the aggression bias depends on
stage of speciation. Indeed, the response ratios of the hybridizing-species populations were significantly different from those in both the single-species population and the two-species populations. The aggression bias towards blue in the single-species population may bestow an initial fitness advantage upon (red) color mutant males, facilitating their establishment in the population. Aggression biases towards own color may also stabilize co-existence of reproductively isolated sister species, because both species (red has consistent own-type bias, chapter 2) would receive more aggression with increasing relative abundance.

The data are consistent with previous findings. Around Makobe Island we have also studied another blue reproductively isolated species, *P. ‘pink anal’*, that co-occurs with *P. nyererei*. Aggression choice tests in the field using SCUBA diving revealed an own-type aggression bias in *P. ‘pink anal’* (chapter 2), similar to the two-species populations in the present study. Furthermore, in the same study, 10 out of 15 blue males of hybridizing-species populations biased aggression towards red males (chapter 2).

The aggression bias towards conspecifics in reproductively isolated species is in accordance with the findings of Seehausen & Schluter (2004). In a field survey on Lake Victoria cichlid communities, they found that closely related species that occur at the same locality tend to differ markedly in coloration. In the same study it was found that territorial males tend to have territorial neighbors of species that are different in nuptial coloration from themselves (Seehausen & Schluter 2004). These patterns can be explained when individuals among closely related species bias their aggression towards conspecifics and males of phenotypic similar species, exerting negative frequency-dependent selection on male nuptial coloration (Seehausen & Schluter 2004).

Interestingly, the aggression bias towards red intruders of blue territorial defenders in the hybridizing-species populations suggests that during the incipient stage of speciation aggression bias alone is unlikely to stabilize the process. Other aspects of male-male competition may affect co-existence of (incipient) species, such as color-effects on behavioral dominance (chapter 4), and/or differences in intrinsic aggression level. There is evidence that red males were more aggressive than blue males in hybridizing-species populations (chapter 3 & 6). Whether the combined effect of different elements of male-male competition can stabilize a trait polymorphism remains to be tested.

The marked difference in aggression biases between the hybridizing-species populations and the two-species populations can be partially understood by taking into account the degree of competition for females. Gene-flow between red and blue males at the two hybridizing-species populations suggests that males of the two phenotypes compete partly for the same set of females. This may render it less advantageous to concentrate aggression on male of its own color than in a situation where red and blue are fully reproductively isolated species, as is the case in the two-species populations.
In the experiments we tested wild-caught fish. The aggression biases that we measured therefore likely mimic the situation in nature. It is possible that aggression biases not only have a heritable basis but are also shaped by experience. Learning effects are unlikely to explain the difference in aggression bias in the case of the hybridizing-species and the two-species populations since males from both population types are familiar with red males. Yet, in the case of the aggression bias towards blue in males from the single-species population we cannot exclude the possibility that this is the result of a lack of experience with red males. Testing to what extent aggression biases have a heritable basis and are shaped by individual experience during ontogeny provides a fascinating avenue for future research (see chapter 5).

The present study is the first test of a crucial theoretical assumption of models of sympatric speciation by sexual selection (Van Doorn et al. 2004; Seehausen & Schluter 2004). The primary conclusion is that aggression bias varies between stages of speciation. The hypothesized negative-frequency-dependence arising from this bias could occur in the initial and final speciation stage but not in an incipient stage. Therefore, aggression bias alone is unlikely to stabilize the process of speciation.

Acknowledgements

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Direct male-male competition can facilitate invasion of new color types in Lake Victoria cichlids

Peter D. Dijkstra, O. Seehausen, Ton G. G. Groothuis

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Abstract

The possibility that disruptive sexual selection alone can cause sympatric speciation is currently a subject of much debate. The initial difficulty for new and rare ornament phenotypes to invade a population, and the stabilization of the resulting polymorphism in trait and preference makes this hypothesis problematic. Recent theoretical work indicates that the invasion is facilitated if males with the new phenotype have an initial advantage in male-male competition. We studied a pair of sympatric incipient species of cichlids from Lake Victoria, in which the red \((P. nyererei)\) and blue males \((P. Pundamilia)\) vigorously defend territories. Other studies suggested that red phenotypes may have repeatedly invaded blue populations in independent episodes of speciation. We hypothesized that red coloration confers an advantage in male-male competition, assisting red phenotypes to invade. To test this hypothesis, we staged contests between red and blue males from a population where the phenotypes are interbreeding morphs or incipient species. We staged contests under both white and green light condition. Green light effectively masks the difference between red and blue coloration. Red males dominated blue males under white light, but their competitive advantage was significantly diminished under green light. Contests were shorter when color differences were visible. Experience of blue males with red males did not affect the outcome of a contest. The advantage of red over blue in combats may assist the red phenotype to invade blue populations. The apparently stable co-existence of red and blue incipient species in many populations of Lake Victoria cichlids is discussed.
Introduction

The possibility of sympatric speciation, in which reduction of gene-flow between emerging species occurs in the absence of geographical isolation, has become increasingly accepted in the past decade (Bush 1994; Seehausen & Van Alphen 1999; Schluter 2001; Via 2001; for recent review see Coyne & Orr 2004). There is now broad consensus that reproductive isolation can arise as a pleiotropic consequence of disruptive natural selection on traits associated with competition for resources. In this view of sympatric speciation, reproductive isolation results mainly from ecological causes, and sexual selection only plays a supporting role (Panhuis et al. 2001; Van Doorn & Weissing 2001; Arnegard & Kondrashov 2004). A growing number of speciation models, many of which were motivated by the explosive speciation of haplochromine cichlids in Lakes Victoria and Malawi, suggest that divergent sexual selection alone can cause sympatric speciation. (Lande & Kirkpatrick 1986; Turner & Burrows 1995; Payne & Krakauer 1997; van Doorn et al. 1998; Higashi et al. 1999; Kawata & Yoshimura 2000; Van Doorn & Weissing 2001). These models are inspired by comparative studies, which indicate that closely related species often differ markedly in their secondary sexual characteristics and mate preferences, and only little in ecology (Bouton et al. 1997; Seehausen & Bouton 1997). However, empirical studies are few (Seehausen et al. 1999b; Endler & Thery 1996; Wells & Henry 1998) and it is difficult to say whether sexual selection is the primary cause of their phenotypic divergence, or whether sexual selection exerting mate choice was recruited by disruptive natural selection (Turelli et al. 2001; Arnegard & Kondrashov 2004; Kirkpatrick & Nuismer 2004). A fundamental problem in sympatric speciation driven by sexual selection is the initial hurdle for a novel rare male phenotype to invade a population against the predominant female mate preference (Van Doorn et al. 2004; Seehausen & Schluter 2004). Successful invasion requires a fitness advantage for a new morph, but maintenance of polymorphism requires that this advantage is negative frequency-dependent (Van Doorn et al. 2004). When exerting selection on the same traits that determine female mate choice, male-male competition for access to spawning territories could generate such frequency-dependence in sexual selection. An advantage could emerge if the new morph induces submissive behavior in opponents, e.g. by being more brightly colored. This would generate negative frequency-dependent selection, because as the frequency of the new morph increases, the proportion of fights of new morphs with ancestral morphs as opposed to new morph males decreases. The symmetry of frequency-dependent selection may be modulated if the ancestral type males change their behavior as a result of social experience with, or genetic adaptation to males of the new morph. Here we experimentally investigate whether the invasion of a new morph may be facilitated by direct male-male competition, using two closely related incipient species of rock-dwelling cichlids from Lake Victoria.
Lake Victoria’s flock of endemic haplochromine cichlid fishes is among the most rapidly speciating groups of vertebrates (Johnson et al. 1996; Seehausen 2002), and is considered an outstanding example of explosive evolution and adaptive radiation (Greenwood 1981; Kaufman 1997; Seehausen et al. 2003). Sexual selection by female choice on conspicuous male color patterns has been proposed as an important factor in the rapid speciation (Dominey 1984; Seehausen et al. 1997; Seehausen & Van Alphen 1998). Males of many of the speciose genera of cichlid fish in Lake Victoria vigorously defend territories to secure mating opportunities, and it appears that possession of a territory is a prerequisite for access to spawnings. The genus *Pundamilia* is a good example of this pattern. Females provide extensive mouth broodcare and preferentially mate with males that hold large territories (Maan et al. 2004). Hence, competition over territory sites is likely intense, and may play an important role in sexual selection (Fryer and Isles 1972; Seehausen et al. 1998b).

We experimented with a pair of hybridizing or incipient species of the genus *Pundamilia*, consisting of a *P. nyererei* (Witte-Maas & Witte 1985)-like form with red and a *P. Pundamilia* (Seehausen et al. 1998a)-like form with blue male nuptial coloration. Incipient species refers to two populations which are not reproductively isolated, representing an early stage of speciation. We used a population (Kissenda Island) in which red and blue phenotypes are undifferentiated at neutral microsatellite loci (M. Taylor et al. unpublished data). In this population, therefore, red and blue males have a largely homogeneous genetic background except for traits that appear to be under disruptive selection. This makes them a suitable system to investigate the effects of nuptial coloration on behavior. Assortative mating among *Pundamilia* sp. is based on female mating preferences for male nuptial coloration, but is constrained by water clarity (Seehausen & Van Alphen 1998). Kissenda Island has turbid water, probably explaining the porous reproductive isolation between the red and blue forms.

Blue phenotypes have a wider and more continuous geographical distribution than red phenotypes. Red always co-occurs with blue, and the latter has the highest record of sympatric occurrences with other members of the *Pundamilia* complex (Seehausen 1996; Seehausen & Van Alphen 1999). This suggest that it is likely that blue populations have been invaded repeatedly by red forms during independent episodes of speciation (Seehausen 1997; Seehausen & Van Alphen 1999; Seehausen & Schluter 2004). If the red nuptial phenotype tends to be the invading phenotype, it requires an initial selective advantage. This advantage would diminish as the frequency of red males increases, because the proportion of fights between blue and red contestants drops. The advantage would also diminish when the behavior of blue males changes with more frequent experience with red males. Such negative frequency-dependent selection could promote a stable color polymorphism. To test the effect of nuptial coloration on the outcome of competitive interactions we staged contests between red and blue males under white and under green light. Green light effectively masks the differences
between red and blue coloration. To test whether prior experience with red males affects competitive ability in blue males, blue males were either given experience with red males or not.

**Methods**

**Fish**

*P. nyererei* and *P. Pundamilia* are morphologically highly similar haplochromines that at many places in Lake Victoria behave like behaviorally isolated sibling species (Seehausen 1997; Seehausen & Van Alphen 1998). Where the lake waters are extremely turbid though, single populations of *Pundamilia* are found, with variable male coloration but no well-differentiated red and blue phenotypes. At intermediate turbidity, differentiated red and blue nuptial phenotypes co-exist as incipient species with less frequent intermediate phenotypes. Such is the population from Kissenda Island in the central Mwanza Gulf in Tanzania. Males of both phenotype classes have blackish underparts and blackish vertical bars on the flanks. Males of the red (*P. nyererei*-like) phenotype, however, are crimson dorsally, yellow on their flanks, and have a crimson dorsal fin. We refer to them as ‘red males’. Males of the blue (*P. Pundamilia*-like) phenotype are greyish white dorsally and on the flanks, and have a metallic blue dorsal fin. We will refer to them as ‘blue males’. At Kissenda Island blue and red occur syntopically (Seehausen 1997).

Males and females were collected at the island and brought to the Zoological Laboratory in Haren in June 2001. We used first-generation offspring bred from this stock. For each phenotype, males were bred from five wild-caught females and three wild-caught males. Experimental males were selected from 60 red and 51 blue males. All males were sexually mature and at least 11 months of age.

**Housing**

Wild-caught males and females were kept in monospecific groups of 13-25 individuals per aquarium (100-300 liter). First-generation offspring were bred from this stock, and reared in sibling groups. The sides and the back of all aquaria were covered with black plastic. All aquaria were connected to a central biological filter system and water circulated continuously. Water temperature was kept at 25 ± 2°C and a 12:12 h light:dark cycle was maintained. All aquaria contained a substrate of gravel. The fish were fed flake food (TetraMin Tropical Fish Flakes) six times per week.

**Experiment**

Experimental males were housed in groups of 20-60 individuals in 100-300 liter aquaria. To avoid the effect of previous encounters in the raising tanks, males were individually housed for at least one week prior to a contest in tanks of 100 liters,
divided in 8-10 compartments by PVC sheets. To avoid social deprivation and to encourage territoriality, all males were kept in compartments with another male visible behind a transparent PVC sheet. Each compartment contained a PVC tube that the fish used as a hiding place. They often indulged in boundary fighting with their territorial neighbor.

Experimental pairs were matched for weight, and had never been housed in visually adjacent compartments during the pre-experimental period. To test whether coloration determines contest outcome, we conducted the experiment both under white light and under green light (see below). Green light eliminates the color difference between red and blue males. Figure 1 illustrates the experimental design. For each light treatment we selected pairs, such that most males experienced one contest under white light and one under green light. To control for possible sequence effects we used a cross-over design, testing some individuals (group A) first under white light, and thereafter under green light, and the other individuals in reverse sequence (group B). We staged 42 contests under white light, and 42 under green light. Two contests from the white light treatment were excluded because we were unable to assign a winner and a loser. Thus, in group A we ended up with 31 contests under white light, and 27 contests under green light. Group B included 15 contests under green light, and nine under white light. To further reduce the effect of prior experience we allowed approximately three months between contests, during which males were housed in large mixed-species aquaria, one for group A (63 fish), and one for group B (24 fish). We ensured that males never fought twice under the same light condition. When composing pairs of males for their second contest it was possible to choose the opponent of one male from at least 5 but often more individuals of which only one could have been the fish that had been the opponent in the previous fight. It is therefore unlikely that re-confrontations with the same male is an important confound in this study. We therefore treated each contest as an independent event.

Monospecific- and Mixed-Species-Housing Treatment

To test the effect of prior experience with red males on competitive ability in blue males, we housed the males before the contest experiment as follows. Prior to the first series of contests under white light (group A, Figure 1), mature males were removed from their sib groups and housed for at least 78 days in either a monospecific- or a mixed-species group in aquaria of 170 liters. We created two monospecific aquaria: one for 36 blue males, one for 36 red males. The mixed-species aquarium contained 19 blue and 17 red males. During this period males were already mature and frequently engaged in aggressive interactions. During the individual housing period prior to the contest, males from the monospecific groups were given a conspecific neighbor, whereas the males from the mixed group were given a heterospecific neighbor.
Males that experienced their first contest under green light were also housed in either monospecific- or mixed-species groups. In the present study, we only analyzed the effect of social experience on the contests under white light.

Twenty blue males from the monospecific group fought against 20 red males from the monospecific group. Eleven blue males from the mixed group fought against 11 red males from the mixed group.

**Light Treatment**

We used green filters to eliminate the color difference between red and blue males. Illumination was provided by 58 W fluorescent light tubes (cool white) in metal hoods. White light was created by wrapping two layers of neutral filters (Filter Neutral Density, code 209 0.3ND, Lee Filters) around the hoods. Green light was created with a single layer of green sheets (Filter Primary Green, code 139, Lee Filters). Light intensity did not differ between light treatments (independent sample t-test: white light: X ± SD = 429 ± 210 lux, N=40, green light: X ± SD = 373 ± 153 lux, N=42, t_{82}=1.351, P=0.18).

**Experimental Procedure**

Males were quickly weighed in air on an electronic pan balance. The weight asymmetry between paired males was calculated as ln (weight red male/weight blue male) following Enquist & Leimar (1983). Weight asymmetries were balanced.
and roughly symmetrically distributed over the two species (absolute weight asymmetry range 0-0.3). We staged contests by placing pairs of males in test aquaria (100 liters). Males were separated by an opaque partition in the middle. Each compartment contained a PVC tube that provides a shelter. A mixture of three to four juvenile fish of either one or both species, showing no nuptial coloration, were placed in each compartment to maintain territoriality in the test fish; they were separated from the test fish by a transparent partition. All pairs were placed in the experimental aquarium 1-3 days before the contest took place. At the start of the contest we removed the opaque partition between the two experimental fish, and the PVC tubes to avoid hiding behavior. The contest was videotaped from behind a blind.

After conflicts had been settled, the fish were separated immediately. Males were blotted dry and weighted again to the nearest 0.1 gram. These final weight measurements were used in the analysis of weight asymmetry and conflict outcome, because these were closer in time to the conflict. Weight asymmetry did not differ significantly between light treatments (independent t-test, \( t_{82} = -0.021, P = 0.98 \)), nor did it differ in favor of either species (white light: \( t_{40} = -0.565, P = 0.58 \); green light: \( t_{42} = -1.551, P = 0.13 \)).

**Observations**

In 82 of the 84 contests that we staged, males engaged in territorial fighting, defined as at least one aggressive interaction in which both contestants performed threat display and/or overt aggression. In all these pairs we could clearly identify a winner and a loser. Males showed the common behavioral repertoire found in cichlid wrestling (Baerends & Baerends-Van Roon 1950; Turner & Huntingford 1986). They usually commenced a fight by raising their fins. This was then followed by several other noncontact displays, such as lateral display, gill display, quivering and tail beating. In several interactions one of the males initially performed courtship display, usually presentation of egg dummies on the anal fin, and the courted male responded to this by showing female mimicry behavior. This was always rapidly replaced by agonistic behavior. Instances of courtship in male-male interactions followed by agonistic behavior has been found in previous cichlid contest studies (Oliveira & Almada 1998). Escalated fighting began abruptly in the form of biting, mouth locking and circling. At the end of each contest one fish would abruptly break from the interaction and flee, accompanied by paling in coloration; this fish was defined as the loser. The contest duration was quantified from the videotapes in seconds. It was defined as the time period between the start of the aggressive interaction, when both fish started to display, and the time the loser started to flee, minus the duration of any pauses and courtship. Pauses were defined as interruptions in contests during which males did not interact with each other. In such cases they showed non-aggressive behavior, such as exploring the tank environment.
Analysis

We tested for the effect of light treatment on contest outcome using binomial, chi-square and Fisher-exact probability tests.

In addition, we used general linear models (GLM), with contest duration as response variable, and logistic regressions, with outcome of contest (red wins or blue wins) as response variable to test the effect of light treatment and monospecific- or mixed-species-housing condition (social experience). Independent variables also included weight asymmetry and contest duration. Models are specified in the results section. We used backward stepwise selection procedures to determine which variables accounted best for the variation in the dependent variable. The criterion to remove a variable was set at $P=0.05$. In the logistic regression we used the Wald test to evaluate the significance of a variable. The fit of a candidate model was tested by comparing the deviance ($D$) of this model with a ‘saturated’ model that contained as many parameters as data points (Hardy & Field 1998).

The variables contest duration and average weight of opponents were \( \ln(x+1) \) transformed to meet the assumptions of parametric analysis.

All tests were two-tailed, and all statistical tests were performed in SPSS 11.0.1 or Statistix 8.

Figure 2 The relationship between weight asymmetry and the probability that red wins, based on the logistic regression with weight asymmetry and light treatment as variables. ——— : White light treatment; - - - - - : green light treatment. Weight asymmetry is expressed as: \( \ln(\text{weight red male/weight blue male}) \). Thus, a negative weight asymmetry indicates that the blue contestant was heavier, zero means that males were equally matched, and a positive value indicates that the red contestant was heavier. Values (blue winner=0, red winner=1) are slightly displaced for visual clarity.
Results

Contest Outcome

The saturated logistic model for conflict outcome consisted of light treatment, WA, contest duration and all interaction terms. The stepwise selection procedure yielded a final model with light treatment (Wald=4.233, \( P=0.04 \)) and weight asymmetry (Wald=8.059, \( P=0.005 \)) as significant predictors of contest outcome. The overall fit of the final model was good (\( D_{79}=81.59, P>0.1 \)). Figure 2 illustrates the probability curve for this final model. This curve shows that the probability that red wins was higher under white light than under green light, and that the outcome was in favor of the heavier fish.

To ask whether males of either color type won more contests, while taking into account weight disparities as well as light condition, we examined contests in which contestants were weight-matched (absolute weight asymmetry less than 0.02, see table 1). Weight-matched red males won significantly more contests than blue males under white light (binomial, \( P=0.03 \)). Under green light the chance of winning was similar for red and blue males (binomial, \( P=0.50 \)). However, the effect of the light treatment on the proportion of contests won by red males in weight-matched contests was only significant at the 10% level (Chi-square: \( \chi^2=3.04, P=0.08 \)).

Contest Duration

Figure 3 shows the contest duration under white and green light. The saturated GLM model for contest duration included the variables light treatment, winner’s color (red or blue), weight asymmetry and all interaction terms. The stepwise procedure selected a final model (\( F_{2,79}=3.108, P=0.050 \)), with the variables light treatment (\( F_{1,79}=4.533, P=0.036 \)) and winner (\( F_{1,79}=2.914, P=0.09 \)) as best predictors of contest duration. Contests under green light were longer than under white light (Figure 3). The winner of the contest was not significantly related to contest duration but we retained this effect in the model because it accounted for some variation (variation within winners of the same color smaller than between winners of different color, as indicated by F-ratio>1). Contest duration tended to be longer when a red male won the contest.

Table 1 Outcome of weight-matched contests with a weight asymmetry (WA) of less than 0.02 for each light treatment. \( P \)-values are given for binomial tests, comparing the number of fights won by each species.

<table>
<thead>
<tr>
<th>Light</th>
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<th>Red wins</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>White</td>
<td>3</td>
<td>11</td>
<td>0.029</td>
</tr>
<tr>
<td>Green</td>
<td>6</td>
<td>7</td>
<td>0.5</td>
</tr>
</tbody>
</table>
**Social Experience**

To determine whether contest duration and/or outcome under white light were affected by social experience we used a GLM and a logistic regression respectively. In the GLM we included apart from social experience the variables winner’s color, weight asymmetry and all interaction terms to predict contest duration. To predict contest outcome we included weight asymmetry, contest duration and all interaction terms in the logistic regression. According to the criteria of the backward selection procedure, social experience was one of the first variables to be removed in both models (logistic regression: social experience \(P=0.88\), GLM: social experience \(P=0.85\)). In addition, a forward regression, starting with social experience did not reveal any significant effect of these variables (logistic regression: social experience \(P=0.56\), GLM: social experience \(P=0.5\)). Thus, social experience is a poor predictor of contest outcome or duration.

![Figure 3 Contest duration (mean and SD) under white (N=40) and green light (N=42). * P<0.05](image)

**Discussion**

*Dominance of Red Males*

We hypothesized that red nuptial coloration provides males with an advantage in male-male competition in the Lake Victoria genus *Pundamilia*, which would facilitate their invasion of blue populations by novel red morphs. We found that
when color differences were visible, red males were more likely to prevail in direct aggressive encounters with weight-matched blue males. Under green light the chance that red or blue won a contest when weight-matched was equal. The difference in outcome of weight-matched contests between light treatments was only significant at the 10% level, which might be attributed to low sample sizes. The logistic regression showed a significant light treatment effect, suggesting that red coloration itself confers an advantage in male-male combat. To the human eye, red males appear much more brightly and conspicuously colored than blue males. If this is also true for the cichlid eye, it is possible that the brightness of red coloration intimidates blue opponents. The observation that contests lasted significantly longer under green light is consistent with this interpretation. It suggests that blue males were more motivated to fight when they could not perceive the red coloration of their opponent.

The advantage of red color in winning a contest is consistent with studies of intraspecific contests in other cichlids and some other teleosts, where redder males tended to have an advantage (Firemouth cichlids *Cichlasoma meeki*: Evans and Norris 1996; Sticklebacks *Gasterosteus aculeatus*: Baube 1997). These authors also manipulated color perception of the males and found a reduction in the chance of winning a contest for redder males when color differences were masked. Rowland and co-workers (1995) suggested that the conspicuousness of red color directly intimidates rivals by stimulating a fear response. The same has been suggested for the effect of bright orange coloration in Midas cichlids, *Amphilophus (‘Cichlasoma’) citrinellum* (Barlow 1983).

Co-existence of red and blue species

The competitive advantage of red over blue males offers a potential explanation for how red males can invade blue populations. However, for red and blue forms to stably co-exist in natural communities it is required that dominant red males are under negative frequency-dependent selection. This could be achieved by blue males becoming less inhibited as a result of fights with the red morph. However, our experiment prior social experience with red males was a poor predictor of contest outcome, suggesting that previous encounters with red males did not improve nor weaken the competitive ability of blue males. Experience of blue males with red males is thus unlikely to counteract the advantage of red males. Nevertheless, red males may be subject to negative frequency-dependent selection, because with increasing frequency of red males, red males would be involved in more contests with other red males in which they lack the advantage that they have in red-blue contests. The advantage of red in male-male combat would then nonetheless still be frequency-independent, possibly leading to a situation in which red males displace blue males. One possibility that promotes co-existence of both morphs is when contests between red males are more costly than fights between blue males, in terms of fight duration and/or risk of injury. This hypothesis remains to be tested.
We observed that heavier males are more likely to win a fight. This has often been shown in cichlid fish (Turner 1994; Neat et al. 1998) but it could be relevant in the context of sympatric divergence and co-existence. The incipient species with a male coloration that conveys a disadvantage in contests may compensate by growing larger body sizes. *P. Pundamilia* males indeed attain a larger size than *P. nyererei* at several islands in Lake Victoria (Seehausen 2006).

Several other factors may further counterbalance the advantage of red males. In a separate study we found that both territorial red and blue males direct more aggressive behavior to red intruders, whereas under green light males did not discriminate between red and blue intruders (chapter 2 & 3). This indicates that redness itself imposes a social cost, and implies that red males are likely to suffer from more aggressive attacks than blue males. Red coloration may also impose a cost due to higher nutritional requirements to maintain red coloration (Candolin 2000), and a higher predation risk of red males to visually guided predators (Endler 1992; Andersson 1994; Godin & McDonough 2003). That red coloration is indeed costly in *Pundamilia*, is suggested by the finding that red color has almost disappeared in many populations in extremely turbid waters with constrained color visibility (Seehausen 1997; Seehausen et al. 1997).

It has recently been suggested that competition for breeding sites may promote diversification and possibly sympatric speciation among haplochromine cichlids (Seehausen & Schluter 2004). Here we described an experimental study in a Lake Victoria cichlid genus in which we showed that male-male competition can assist a novel male phenotype to invade. Our study supports the hypothesis that male-male competition can be a significant facilitating force of sympatric speciation.

**Acknowledgements**

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Learned aggression biases in Lake Victoria cichlid fish

Peter D. Dijkstra, O. Seehausen, R. E. Fraterman, Ton G. G. Groothuis
Abstract

Recent studies suggest that male-male competition for spawning sites can exert negative frequency-dependent selection on a male secondary sexual trait, for example nuptial coloration. This is the case when males bias aggression towards rivals of their own color, conferring a fitness advantage upon rare phenotypes. Such an aggression bias may promote the evolution and maintenance of stable color polymorphisms in the course of speciation. It could operate in the extensive color radiations of haplochromine cichlid fish. In a previous study using wild-caught blue and red *Pundamilia* males from Lake Victoria we measured aggression biases in simulated intruder choice tests. We found that wild-caught blue males from a location where red males are absent, biased aggression towards blue stimulus males, whereas those from a location where blue and red occurred sympatrically, biased aggression towards red stimulus males. Here we test, using lab-bred blue males from both types of communities, the hypothesis that exclusive experience with blue males leads to an aggression bias towards blue, and that experience with blue and red males leads to an aggression bias towards red. Contrary to predictions, blue males without prior experience with red did not distinguish between blue and red males. Blue males with prior experience with blue and red preferentially attacked blue stimulus males. The data suggest that prior experience can affect aggression biases, and that experience with more than one phenotype is required for the development of biases. Yet, our results cannot explain the direction of differences in specific biases observed in wild-caught males from different populations.
Introduction

Effects of learning on developing mating preferences have been intensively investigated. Filial imprinting (Clayton 1988; Oetting et al. 1995), sexual imprinting (Ten Cate & Vos 1999) as well as learning in the adult phase (Bisschop 1994; Mugarran & Ramnarine 2004) may help animals to identify conspecific mates (see also e.g. Bereczkei et al. 2004). Learned mate preferences may also play a role in speciation, e.g. in determining the rate at which premating isolation can arise (Laland 1994; Irwin & Price 1999; ten Cate & Vos 1999; Verzijden et al. 2005). Few studies on learning concern intrasexual interactions (Vos 1994; Hansen & Slagsvold 2003; Engeszer et al. 2004). Since intrasexual selection is increasingly recognized as a diversifying force (Seehausen & Schluter 2004; Mikami et al 2004; Van Doorn et al. 2004; this thesis), learned aggression biases may likewise play a role in the process of speciation.

Research on sympatric speciation by sexual selection has largely focused on the role of female mate preference in driving changes in male phenotypes (Turner & Burrows 1995; Payne & Krakauer 1997; Higashi et al. 1999; Kawata & Yoshimura 2000; Arnegard & Kondrashov 2004). Van Doorn and co-workers (2004) indicated that intrasexual selection can be a previously neglected essential ingredient to the process. If males bias aggression towards like-colored phenotypes, rare males would receive less aggression and may consequently enjoy elevated fitness (Mikami et al. 2004; Seehausen & Schluter 2004; Van Doorn et al. 2004). The negative frequency-dependence that is expected to arise from such an own-type bias in aggression might promote the spread of novel male phenotypes and the stabilization of color polymorphisms, thereby facilitating sympatric speciation. A previous study showed color-based aggression biasing in a Lake Victoria cichlid fish (chapter 2). Although aggression biases are likely to have a heritable basis, learning effects may modulate the strength of own-type biases. For example, an ancestral morph may learn to weaken its own-type bias in aggression when the novel morph increases in relative abundance. Such learning would explain differences in own-type aggression bias observed among populations of haplochromine cichlid fish from Lake Victoria (chapter 2 & 3). The aim of the present study was to test the role of learning in shaping aggression biases in Lake Victoria cichlid fish.

Lake Victoria’s flock of endemic haplochromine cichlid fishes is among the most rapidly speciating groups of vertebrates (Johnson et al. 1996; Seehausen 2002; Kocher 2004), and is a textbook example of explosive evolution and adaptive radiation (Greenwood 1981; Kaufman 1997; Seehausen et al. 2003). The mating systems of the most species rich genera suggest that both inter- and intrasexual selection on male nuptial dress may be a potent diversifying force (Dominey 1984; Seehausen et al. 1997; Seehausen & Van Alphen 1998; Maan et al. 2004; Seehausen & Schluter 2004; this thesis). In their lek-like mating system, parental investment is strongly skewed towards females (Seehausen 1996). Males vigorously defend permanent territories, and it appears that possession of a territory can be a
prerequisite for mating success (Maan et al. 2004). Females use territory quality cues for assessing prospective mates (Maan et al. 2004; chapter 7). Hence, competition over territory sites is likely intense (Fryer & Isles 1972; Seehausen et al. 1998b), and may be an important component of sexual selection (Seehausen & Schluter 2004).

In this study we tested the role of learning in shaping aggression bias in blue male phenotypes of the *Pundamilia complex*, endemic to Lake Victoria, towards either red or blue intruding phenotypes. Red and blue *Pundamilia* phenotypes represent a common color dichotomy among closely related species of haplochromine cichlids (Seehausen et al. 1999a). The blue phenotype is distributed throughout the lake, whereas the red phenotype shows a patchy distribution nested within that of blue (Seehausen 1996; Seehausen & Van Alphen 1999). This may suggest that blue nuptial coloration represents the ancestral state and that blue populations have been invaded repeatedly by red forms during independent speciation events (Seehausen 1997; Seehausen & Van Alphen 1999).

In a previous study using wild-caught blue and red *Pundamilia* males we measured aggression biases in simulated intruder choice tests (chapter 4). Blue males occurring sympatrically with red biased aggression towards red rivals, whereas those from a location without a red population biased aggression towards blue. This difference between the populations may be heritable, or it may be attributable to the difference in experience with the red phenotype. The learning hypothesis predicts that aggressive experience with only blue males would lead to a bias in aggression towards blue males, whereas experience with both blue and red males would lead to a bias towards red males. We used first generation lab-bred blue sons of wild-caught blue males originating from locations where they occur sympatrically with red, and from one location where the red species is absent.

**Methods**

**Species**

We studied lab-bred fish the parents of which originated from two kinds of populations. The first were two populations where ‘red’ and ‘blue’ *Pundamilia* are incipient species (referred to as ‘incipient-species population’ type). The second was a population of only one *Pundamilia* species most males of which are blue (referred to as ‘single-species population’ type).

The *incipient-species populations* from Python and Kissenda Islands in the Mwanza Gulf of Lake Victoria (Tanzania), consist of a red incipient species, resembling *Pundamilia nyererei* (Witte-Maas & Witte, 1985) and a blue incipient species, resembling *Pundamilia Pundamilia* (Seehausen et al., 1998a) from sites with no hybridization. We use the term ‘incipient’ because the occurrence of some intermediate forms between red and blue around Kissenda and Python Islands suggests some hybridization (Seehausen 1997; unpublished data), resembling an
incipient stage of speciation (chapter 2 & 3). Males of *P. nyererei* are crimson dorsally, yellow on their flanks, and have a crimson dorsal fin. We refer to them as ‘red’. Males of *P. Pundamilia* are greyish white dorsally and on the flanks, and have a metallic blue dorsal fin. We refer to them as ‘blue’. Both are confined to rocky shores and islands in Lake Victoria (Seehausen 1996). Kissenda and Python males are phenotypically indistinguishable and wild-caught males have similar aggression biases (chapter 2 & 3).

Fish from the *single-species population* were collected around Luanso Island, Lake Victoria (Tanzania). Most males from this population are similar to *Pundamilia Pundamilia*, some are intermediate between the blue and the red phenotype, but fully red males have not been reported (Seehausen 1996; 1997). We hence refer to Luanso males as ‘blue’.

**Housing**

We bred fish from wild-caught males and females in single-species aquaria. Individual sib-groups were housed in 50-100L aquaria and guarded by their mother for one week. The sides and the back of the aquaria were covered with black plastic sheets. All aquaria were connected to a central biological filter system and water circulated continuously. Water temperature was kept at 25 ± 2°C and a 12:12 h light:dark cycle was maintained. All aquaria contained a gravel substrate. The fish were fed flake food (TetraMin Tropical Fish Flakes) once per day.

**Blue- and mixed-housing treatment**

When starting the experiment, we randomly assigned fish (standard length range 29.3-63.8 mm) to one of the two treatments (explained below). Both treatments contained several aquaria each housing a group of eight fish. When assigning the fish to the two treatments, they were at least six months of age when nuptial coloration started to develop. Prior to treatment allocation fish had been raised in sib-groups and were, hence, naive with respect to the red phenotype. Fish were selected from 10 sib-groups of incipient-species populations and 5 of the single-species population. Each treatment group (each housed in 110-170L aquaria) had the following features. First, males from the three populations were never used together in the same treatment group. Second, since allocation to treatment groups was randomized with respect to sib-group, the majority of males did not have sibling in their treatment group. Finally, each treatment group consisted of three to five males, supplemented to eight individuals with females. For the incipient-species populations we created 10 treatment groups per treatment and for the single-species population we created 5 treatment groups, so that there was a total of 30 treatment groups or aquaria.

Blue-treatment groups were housed between two blue neighboring males, whereas each mixed-treatment group was positioned between one blue and one red neighboring male. Neighboring males (lab-bred *Pundamilia* males from Kissenda
Island SL range 72.0-90.1 mm, N=60) were individually confined at both ends of an aquarium, using perforated (diameter 23 mm) transparent sheets. The perforations allowed treatment-group fish to move between compartments. Thus, each treatment group aquarium consisted of a central compartment for the treatment-group fish and two side compartments. The perforated sheets ensured that neighboring males remained in their compartments, while allowing treatment-group fish to see and physically interact with the neighboring males. Neighboring males in each treatment group were matched for standard length: the difference was less than 2.2%, measured as\((100\times (L-S))/S\), where \(L\) = the longest and \(S\) = the shortest fish. Neighboring males were larger than the treatment-group fish, and they were always in territorial condition, as indicated by dark vertical bars. Territoriality of the neighboring males was further ensured by providing them with a PVC tube for shelter (see figure 1). They would usually expel intruding treatment-group fish from their compartment. This set-up standardized dominance relationships among treatment groups, since each group consisted of one dominant treatment-group fish (by necessity a blue phenotype), and two dominant neighboring males. Males were kept three months long in the treatment groups before they were tested for aggression biases. In case a treatment-group fish died (mortality was 0.9 fish/treatment group during the treatment housing), we replaced the fish by a female \(P. \text{Pundamilia}\) from Kissenda Island to ensure that group size remained the same.

After the three months treatment, treatment-group males (now referred to as test males, standard length range 42.4-74.7 mm) were individually housed for at least a week prior to transfer to the test aquarium in which the aggression bias was measured. During the individual housing, males were kept in separate compartments, each with a PVC tube refuge and with visual contact to one blue male through a transparent plastic sheet. The individual housing ensured territorial condition but avoided unwanted effects of social isolation. For the incipient-species population type we tested 19 blue-treated males (9 from Kissenda Island and 10 from Python Island) and 21 mixed-treated males (11 from Kissenda Island and 10 from Python Island). For the single-species population type we tested 20 blue-treated males, and 18 mixed-treated males.

**Aggression choice test**

We used the same test paradigm as described elsewhere (chapter 2 & 3). Test males were transferred from their individual compartment to the test aquarium one day before a trial to allow them to acclimatize. A PVC tube was provided as a refuge. A test aquarium consisted of a larger experimental compartment (55 x 35 x 39 cm, l x w x h) with a PVC tube for the test fish, and a smaller compartment (5 x 35 x 39 cm) for a neighboring fish. The neighboring fish was a small \(P. \text{Pundamilia}\) male (Kissenda/ Python Island, first generation offspring) needed to maintain territorial condition of the test fish. The neighboring male was separated from the test fish by a transparent partition. At the start of a trial, size-matched (standard length) blue
and red stimulus males confined in watertight transparent tubes were simultaneously placed in the compartment 20 cm apart. The test males perceive these stimulus males as intruders, and typically respond aggressively to both stimulus males, whilst the small neighboring fish is neglected. Directly after introduction of stimulus males the trial was recorded on videotape for 5 minutes. Between trials we alternated the left and right position of the stimulus males with respect to color.

Stimulus males consisted of first generation lab-bred red and blue males from Kissenda or Python Island. Kissenda and Python males are indistinguishable. We have shown previously using light manipulation experiments, that aggression biases in these populations are based on differences in nuptial coloration rather than on other traits of the males (chapter 2). Stimulus pairs (N=21) were formed by matching a blue and a red male for standard length: the difference was less than 4.9%. Each stimulus fish was used only once per treatment-population type combination, except for four red and four blue males. These fish were used in two pairs, but when using a male for the second time, it was never paired with the same male as before (Kodric-Brown & Johnson 2002; Pauers et al. 2004). Before the trials, stimulus males were kept in separate compartments within which a PVC tube was placed as a refuge and with visual contact to a red or blue male through a transparent plastic sheet for at least a week.

Analysis

We scored the number of attack behaviors to the blue stimulus male ($A_b$) and the red stimulus male ($A_r$) from the videotapes (Baerends & Baerends-Van Roon 1950; chapter 2 & 3). Tape analysis was carried out blindly with respect to population and treatment. The aggression bias, expressed as the attack ratio, was the fraction of all attacks of a male that was directed towards the blue stimulus male [$A_b/(A_r+A_b)$]. The attack ratio data were arcsine square root transformed. The level of aggression is expressed as the total number of attacks to both stimulus males during the five minute trial period.

We first tested for associations between sib-group and attack ratio, using linear hierarchical modeling in MLWin 2.0. We tested on average 2.6 males per community (standard error ± 0.2, median = 2, range 1-5). The possible non-independence of individual test fish from the same treatment group explained none of the observed variation in the attack ratio (P>0.5). In further analysis we treated each male as an independent case. The aggression bias in the two incipient-species populations did not differ significantly in either treatment (blue treatment: $t_{17}$=1.279, $P$=0.2; mixed treatment $t_{19}$=-1.440, $P$=0.2). Hence, we pooled the data. All other tests were performed in SPSS 12.0.1. We tested for the effect of treatment and population type (both two levels) on the attack ratio by ANOVA. We evaluated aggression bias for each treatment separately by testing whether the attack ratio deviated from 0.5 using a repeated measure ANOVA with population type as explanatory variable.
All reported probabilities are for two-tailed tests of significance. We report means and ± standard errors.

**Results**

Males directed aggressive behaviors to both types of stimulus males. On average, males performed 42.6 ± 2.0 attacks/5 min.

Figure 1 shows the attack ratio of blue-treated and mixed-treated males from the single-species population and the incipient-species population type. Blue-treated males showed no aggression bias: their attack ratios did not deviate from 0.5 (Figure 1, RM-ANOVA, deviation from 0.5: F$_{1,38}$=0.435, P=0.5). This lack of a bias was observed both in males from the single-species and males from the incipient-species population types (population type effect: F$_{1,37}$=0.12, P=0.7). By contrast, mixed-treated males showed a bias for the blue stimulus male: their attack ratio deviated highly significantly from 0.5 (Figure 1, RM-ANOVA, deviation from 0.5: F$_{1,38}$=11.807, P<0.001). Also this bias did not differ between population types (F$_{1,37}$=0.021, P=0.9). After removing population type (F$_{1,75}$=0.018, P=0.9) and
the interaction treatment x population type (F_{1,74}=0.118, P=0.7) from the model, we found a significant treatment effect on attack ratio (ANOVA, F_{1,76}=4.177, P<0.05). This suggests a role for learning in shaping aggression bias (Figure 1) and that the effect was similar for both population types.

Post-hoc tests revealed no significant treatment effect for the two population types separately (independent t-test: incipient-species population type t_{38}=1.542, P=0.1; single-species population type t_{36}=1.311, P=0.2), probably due to a lack of power. Further, we evaluated aggression biases for each population type and treatment combination separately testing attack ratios against 0.5 with one-sample t-tests. The attack ratios did not deviate significantly from 0.5 in males from the blue treatment (incipient-species population type t_{19}=-0.690, P=0.5; single-species population type t_{20}=-0.213, P=0.8). Attack ratios deviated significantly from 0.5 in males from the mixed treatment (incipient-species population type t_{17}=3.001, P<0.01; single-species population type t_{20}=-2.192, P<0.05).

Discussion

Male-male competition may facilitate the invasion of novel or rare male phenotypes and maintain stable co-existence of incipient species through negative frequency-dependent selection (Mikami et al. 2004; Seehausen & Schluter 2004; Van Doorn et al. 2004; chapter 3). Frequency-dependence may arise when territory defenders bias aggression towards similarly-colored rivals. We tested whether aggression bias can be modulated by learning, and whether a different social background could explain the differences observed among populations. We observed indeed a significant effect of learning: exclusive experience with blue males resulted in lack of aggression bias, whereas experience with blue and red males resulted in an aggression bias towards blue males. Wild-caught fish of the different populations do differ in aggression bias (chapter 2 & 3), but not the lab-bred fish in this study. This contrast indicates that the difference in aggression bias in wild-caught males might be attributable to learning and differences in experience.

Although the evidence for learning supports our main hypothesis, the results do not support our predictions concerning the direction of the effect. Predictions stemmed from aggression biases of wild-caught males measured in a previous study (chapter 2 & 3): a bias towards blue in the single-species population where red males are absent, and a bias towards red in blue males from incipient-species populations where they co-occur with red. We can only speculate about the possible reasons why the direction of the treatment effect is opposite to the expectation. First, our treatment was not likely to mimic the situation in the wild for either population type. For example, in the incipient-species populations red phenotypes are the most abundant *Pundamilia* form (Seehausen 1997; unpublished), a situation not reflected in our mixed treatment. Secondly, in the wild these males experience both color types from an early age onwards, whereas we specifically looked at the effect of learning in sexually mature fish. Finally, males from the single-species population do not only experience competition with blue
conspecifics, but also with males of other species some of which have different nuptial coloration. Our results suggest that experience with more than one color phenotype may be required to develop any aggression bias.

From a functional perspective, learning might be important to recognize competitors for mating opportunities (Hansen & Slagsvold 2003) and allows individuals to adapt quickly to changing environments (Immelmann 1972; Bolles & Beeher 1988). To what extent this applies to learned aggression biases in haplochromine cichlid fish in the wild is a topic that deserves more empirical and theoretical investigation. Learning is theoretically an effective way of generating own-type biases in aggression in newly arising species, because they do not require linkage disequilibria to form between alleles causing different colors and alleles causing different aggression biases. In theory, learning may thus modulate the strength and symmetry of frequency-dependent selection generated by male-male competition.

In conclusion, this study shows that aggression biases may at least partially be acquired by learning in an animal species with strong inter- and intrasexual selection on the male secondary sexual traits. Our findings may have implications for understanding the role of male-male competition in speciation and species co-existence.

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Male-male competition in single-color and mixed-color assemblages of cichlid fish: understanding species co-existence and diversification via intrasexual selection

Peter D. Dijkstra, Charlotte Hemelrijk, Ole seehausen, Ton G. G. Groothuis
Abstract

The co-existence of ecologically similar species is a classical puzzle in ecology. It has been proposed that co-existence can be facilitated through male-male competition for mating territories. Negative frequency-dependent selection on male secondary sexual characters is generated when male aggression is primarily directed towards similar rather than towards dissimilar phenotypes. We studied experimental assemblages of cichlid males to test whether co-existence of two color phenotypes could be facilitated through male-male competition. We used blue and red males of two ecologically similar and fully sympatric color morphs of the Lake Victoria cichlid genus *Pundamilia*, resembling *P. Pundamilia* (blue) and *P. nyererei* (red). A previous investigation showed that red males dominate blue males in dyadic interactions. Yet, in Lake Victoria, red populations of *Pundamilia* as a rule occur sympatrically with at least one blue species, suggesting that red males cannot displace blue males through male-male competition. Here we investigated male-male competition in assemblages of territorial males in large aquaria. Males were allowed to form a dominance hierarchy in assemblages composed of either only red males, only blue males (single-color assemblages), or males of both colors (mixed-color assemblages). We found that (1) in mixed-color assemblages none of the morphs dominated the other one, in contrast to the situation in dyadic interactions, that (2) an own-type bias in aggression is expressed in mixed-color assemblages, that (3) the level of aggression was higher in red assemblages than in blue assemblages, and that (4) co-existence in mixed-color assemblages suppressed the level of aggression in red males. We conclude that male-male competition for territory space may be a significant force in speciation and species co-existence. Our data also show that studies on dominance may yield different results depending on whether a group set-up or a dyadic set-up is used.
**Introduction**

The cichlid fishes of the Great African Lakes are renowned for their adaptive radiation and explosive speciation (Kocher 2004). Lake Victoria is the youngest of the Great African Lakes (0.25-0.75 Myr; Fryer 1996) which is believed to have largely dried up as recently as 14500 years ago (Johnson et al. 1996; 2000). Although some of the species diversity may have persisted in refuges during this period (Seehaus 2002; Verheyen et al. 2003) most of the diversity likely evolved in an extremely short time. Their astounding diversity has motivated many theoretical and empirical investigations of phenotypic divergence and maintenance of species diversity (e.g. Turner & Burrows 1995; Higashi et al. 1999; Van Doorn et al. 2004; Maan et al. 2004; 2006a,b,c; Haessler & Seehaus 2005; for reviews see: Kocher 2004; Genner & Turner 2005; Seehaus 2006a). Their evolutionary plasticity has promoted adaptive radiation of cichlids (Liem 1973; Galis & Drucker 1996; Albertson et al. 2003; Seehaus 2006a). Many closely related sympatric species, however, are anatomically and ecologically similar, while exhibiting striking variation in male nuptial coloration (Seehausen & Van Alphen 1999). Lack of gene flow between closely related species is often due to female mate choice based on male nuptial coloration (Seehausen & Van Alphen 1998; Knight & Turner 2004). The mechanisms underlying ecological co-existence of such species has long been recognized as an unresolved problem (e.g. Fryer & Iles 1972; Reinthal 1990; Genner et al. 1999a; Genner & Turner 2005). It has recently been proposed that aggressive interactions between males may promote diversification into, and co-existence of ecologically identical species (Mikami et al. 2004; Seehausen & Schluter 2004). In haplochromine cichlids, adult males vigorously defend long-term mating territories to secure spawnings (Parker & Kornfield 1996; Maan et al. 2004). Competition over territories is intense, and is likely to exert sexual selection. If territorial males bias aggression towards phenotypically similar rivals, rare male phenotypes would receive less aggression than common male phenotypes. Such a bias in aggression is expected to yield negative frequency-dependent intrasexual selection on male nuptial dress. It would confer a fitness advantage to males of a rare phenotype relative to males of the more abundant phenotype, thereby promoting invasion of either color type and facilitating co-existence of male color morphs and incipient species (Mikami et al. 2004; Seehausen & Schluter 2004). More intense aggression between similar than between dissimilar males is consistent with field data on Lake Victoria cichlid communities. Seehausen & Schluter (2004) found (1) that closely related species that occur at the same locality differ significantly more often in male nuptial coloration than males of randomly chosen species; and (2) among two closely related and ecologically similar species within a site, males tended to have territorial neighbors with different nuptial coloration. The latter pattern was also observed in cichlids from Lake Tanganyika (Kohda 1998). Negative frequency-dependent selection generated by own-type biases in aggression would also have
significant bearing on the likelihood of sympatric speciation by mate choice (Van Doorn et al. 2004; this thesis).

In a given two-species assemblage males encounter fewer conspecifics and thus have less need to behave aggressively than in a single-species assemblage. Therefore, in terms of general aggression level, and the costs of establishing territories, the mean fitness of males would be higher in a two-species assemblage than in a single-species assemblage at identical total densities. This predicts that less diverse communities are open to invasion by novel color types.

In this study we investigate aggressive behavior in red and blue males in experimental assemblages. We used a pair of hybridizing incipient species of the genus *Pundamilia* from Kissenda Island, consisting of males resembling *P. nyererei* (Witte-Maas & Witte 1985) with red and others with blue nuptial coloration resembling *P. Pundamilia* (Seehausen et al. 1998a). Red males have a chance of 85% to win a combat with a weight-matched blue male in dyadic interactions. This is largely an effect of red color itself: we have shown that the advantage of red males disappears under green light (chapter 4). The geographical distribution in Lake Victoria suggests that the red phenotype does not displace the blue phenotype, because red *Pundamilia* always occurs sympatrically with blue *Pundamilia* (Seehausen & Van Alphen 1999). Yet, at many places, the two are ecologically highly similar (Bouton et al. 1997; Seehausen 1997), raising the question how red and blue sister species can stably co-exist. It was therefore important to validate findings from dyadic interactions trials in experimental assemblages, which better reflects the natural situation where males have interactions with multiple rival males. Dominance relationships between morphs in an isolated pair situation with only a one time interaction do not necessarily reflect those between both morphs in a multiple male assemblage that is maintained over a longer time span. This is because dominance relations result not only from fixed traits, but also through self-reinforcing effects of winning and losing conflicts among assemblage members (Chase et al., 2002; 2003; Hemelrijk & Wantia, 2005).

We studied aggression and social hierarchies among males in mixed-color (blue and red males together) and single-color (either only red or only blue) assemblages during three consecutive days. We evaluated dominance relationships between red and blue, aggression biases and aggression level. In the mixed-color assemblages, we tested which color type dominated the other one if any. We also tested the hypothesis that males of both color morphs exhibit a bias in aggression directed towards male of their own color. If such an own-type bias exists in aggression, we predicted that sharing space by morphs with different colors results in reduced assemblage-wide aggression levels compared to assemblages where all males are of the same color. This is because in a mixed-color assemblage, individuals encounter more individuals with who they share a mutually ‘tolerant’ attitude than in a single-color assemblage (Mikami et al. 2004). By the same token, we predicted that space is available for more males to reach territorial status in a mixed-color assemblage than in a single-color assemblage.
Methods

Fish
We used first-generation offspring from fish collected in June 2001 around Kissenda Island, Lake Victoria, Tanzania. All males were sexually mature and at least 11 months of age.

Housing
Males were reared in sib groups. When the first fish started to mature (at approximately 6 months of age) they were translocated into stock aquaria, containing males and females of both incipient species. The sides and the back of all aquaria were covered with black plastic. All aquaria were connected to a central biological filter system and water circulated continuously. Water temperature was kept at 25 ± 2°C and a 12:12 h light:dark cycle was maintained. All aquaria contained a substrate of gravel. The fish were fed flake food (TetraMin Tropical Fish Flakes) at least once per day, and a mixture of ground shrimps two times per week. We housed males for at least one week prior to an experiment individually in aquaria measuring 90 x 36 x 30 cm (l x w x h), divided into 8 compartments by PVC sheets. To avoid social deprivation and to encourage territoriality, all males were kept in compartments with another male visible behind a transparent PVC sheet. Each compartment contained a PVC tube that the fish used as a hiding place.

We fitted three PVC tubes (∅ 15 cm, length 21 cm) placed vertically to stimulate territoriality in the experimental aquaria, measuring 250 x 66 x 46 cm (l x w x h).

Experiment
We used the following three experimental treatments (the first two are single-color treatments): (i) red (N=7 assemblages): eight red males in one aquarium; (ii) blue (N=7 assemblages): eight blue males; (iii) mixed color (N= 12 assemblages): four red and four blue males together in one aquarium. Fish in all three treatments were approximately size matched within groups. Information about the mean male weight in each assemblage, the variation in male weight in each assemblage, and the mean weight of red and blue males in mixed-color assemblages are summarized in table 1. None of the parameters differed significantly between treatments or morphs (independent t-tests, Ps>0.05).

To reduce the aggression level among experimental males, we used 15 Melanotaenia lacustris (rainbow fish) as background fish in each experimental aquarium. These fish lack bright coloration, are nonterritorial, and are standard background fish in our laboratory facilities.

Most males were used once in the single-color treatment, and once in the mixed-color treatment. The interval between use of the same male was at least 15
days. Half of the males first experienced the single-color and then the mixed-color treatment and the other half vice versa. Prior to a test, we weighed all males to the nearest 0.01 g. We individually clipped males for individual recognition on their dorsal (two positions) and caudal fin (three positions), using scissors (chapter 8).

Table 1: The weight of test fish based on means per assemblage for each assemblage type and colour morph separately. The variation in weight is indicated by the average coefficient of variation (C.V.). The C.V. for each assemblage is calculated as mean weight/standard deviation. Shown are the mean and SE

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Morph</th>
<th>N</th>
<th>Weight</th>
<th>Coefficient of Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>Blue</td>
<td>Blue</td>
<td>7</td>
<td>15.10</td>
<td>1.16</td>
</tr>
<tr>
<td>Red</td>
<td>Red</td>
<td>7</td>
<td>14.04</td>
<td>0.96</td>
</tr>
<tr>
<td>Mixed</td>
<td></td>
<td>12</td>
<td>14.84</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>Blue</td>
<td></td>
<td>14.99</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td></td>
<td>14.70</td>
<td>0.84</td>
</tr>
</tbody>
</table>

Behavioral observations

We observed each assemblage for three consecutive days following release of the fish into the aquarium. Each observation lasted 45 minutes, amounting to a total observation time of 135 minutes per assemblage. The males displayed the common aggressive behaviors of cichlids (Baerends & Baerends-Van Roon 1950). We recorded aggressive interactions that resulted in a displacement, with the fish being displaced defined as loser and the other one as winner. Displacements could come about via (1) attack (overt aggression) and (2) display (covert aggression). These behaviors were recorded by tape recorder. A displacement via an attack was defined as one fish charging or chasing another fish. A displacement via a display was defined as one fish showing lateral or frontal display, followed by fleeing of his opponent, or as display of both fish to each other, followed by one of them fleeing. We also noted every 15 minutes (3 times per observation session) which males had their vertical bars turned on, which is an indicator of territoriality (Maan et al. 2004). Males that had turned on their vertical bars at least once during an observation session were defined as males with territorial status.

Data analysis

Dominance

The dominance of males of one morph over those of the other morph in the mixed-color assemblages was inferred in two ways. Firstly, we compared the number of
red alpha males with the number of blue alpha males. The alpha male is the most dominant male in an assemblage. Secondly, we examined the degree of dominance of red over blue males over all members in an assemblage (standardized U-value, Hemelrijk et al. 2003; submitted).

For determining the alpha male and the degree of dominance of males of one morph we generated dominance matrices for each day separately. To this end, we calculated for each male the average Dominance Index, which is the mean fraction of aggressive interactions won by the male from each of the other males with which the individual interacted (Hemelrijk et al. 2005). The male with the highest average Dominance Index was considered the alpha male. The average Dominance Index allowed us to rank males in each assemblage. To test for morph-specific dominance, we compared the number of red and blue alpha males over the three days for each mixed-color assemblage using a sign-test.

The degree of dominance of red males over blue males in an assemblage was also estimated by the method developed by Hemelrijk and co-authors (Hemelrijk et al 2003; submitted). It is based on the relative dominance of males of one morph over those of the other morph using a Mann-Whitney U statistic (Siegel & Castellan, 1988). For each red male the number of blue males ranking below him was counted. The value of the statistic is calculated as the sum of these countings. Note that for a mixed-color assemblage of eight males (four red and four blue) the maximum value is 4×4=16. The standardized U-value is corrected for the maximum value at this sample size. Thus, it is calculated as the U-statistic of red over blue, divided by the maximum value. A value of 0.5 indicates equal dominance of red and blue males, a value >0.5 dominance of red males and <0.5 dominance of blue males. Complete dominance of red, which implies that all red males are dominant over all blue males, equals 1, and complete dominance of blue equals 0. To test for dominance of red over blue, we tested if the standardized U-value deviates from 0.5, using a repeated measure ANOVA (RM-ANOVA), with day as repeat and nested herein the standardized U-value and 0.5.

**Aggression bias and aggression level**

For the analysis of bias in and level of aggression, we used attacks but not displacements, as attacks represent an unequivocal indication of aggression. Data were calculated at the assemblage level for each day separately.

To evaluate aggression bias we calculated attack ratios, which is the number of attacks to males of own morph divided by the sum of attacks to all males. If there is no aggression bias the attack ratio is 3/7, because a red or a blue male in a mixed-color assemblage had the opportunity to attack four males of the other color, but only three males of its own color. We tested for aggression bias by testing the attack ratio against 3/7, using for each morph RM-ANOVA with day as repeat, and nested herein the response variable attack ratio and the no-bias ratio of 3/7.

For analysis of the aggression level, we focused on both the rate of received attacks and the rate of performed attacks per individual. In terms of costs of male-
male competition, both measures of aggression level are relevant. We tested the hypothesis that aggression is reduced in mixed-color assemblages relative to single-color assemblages. To this end, we used for each morph a RM-ANOVA with day as repeat and response variable either the rate of received attacks or the rate of performed attacks.

Proportions were arcsine square root transformed and levels of aggression were ln (x+1) transformed to meet assumptions of parametric testing. Statistical analyses were carried out with SPSS 12.0.1. All reported probabilities are for two-tailed tests of significance, unless stated otherwise. For RM-ANOVAs we report the effect of day and interactions only when significant. In the RM-ANOVA we tested whether sphericity could be assumed and corrected the degrees of freedom according to the Huynh-Feldt method if this was not the case. Although aggression bias and aggression level were analyzed with RM-ANOVAs, to streamline graphical presentation, we plotted the mean (± SE) based on the average of the three daily scores.

Figure 1 The attacks bias (attack ratio) for own morph, calculated as the number of attacks towards males of own morph divided by the total number of attacks. Shown are the mean and SE based on the average of the daily scores for red males and blue males (N=12). Asterixes indicate significant differences (P<0.05) from the no-bias ratio of 3/7 (dotted line).

Figure 2 Aggression level, expressed as the rate of received and performed attacks per individual per assemblage averaged over the three days. Shown are the mean and SE for each morph in single-color assemblages (black) and mixed-colour (open) assemblages. Note that received and performed attacks are equal in single-color assemblages, but not in mixed-color assemblages. Significant differences are indicated by asterix ((*) P=0.05, *P<0.05, **P<0.01), for statistics see text. Sample sizes are 7 blue, 7 red and 12 mixed-color assemblages).
Results

Dominance

Dominance of one morph over the other: the alpha male

The male(s) with the highest average Dominance Index in an assemblage, defined as alpha male(s), were all in territorial condition, as indicated by dark vertical bars. To test for dominance of males of one morph over males of the other, we examined the number of red and blue alpha males in each mixed-color assemblage on every day (table 2). Most assemblages were dominated by a single alpha male on any one day. We averaged these numbers over the three days, and found no significant difference in the number of blue and red alpha males (paired t-test, \( t_{11}=0.338, P=0.7 \)).

Table 2 Alpha male information: blue and red alpha males for each assemblage and day for mixed-colour assemblages (N=12). Each letter represents one alpha male: on each observation day we usually observed one alpha male per assemblage, except in three cases. The number of times we observed a red and a blue alpha male averaged over the three days is also listed. Based on these averages, we noted whether we counted more red than blue alpha males in an assemblage. Finally, we show the standardized U-values averaged over the three days for each assemblage (mean ± SE). The standardized U-value (U-value) is the mean dominance rank of red males, expressed as the average fraction of blue males ranking below each red male (for full explanation see method section). A value of 0.5 indicates equal dominance for red and blue males, a value >0.5 dominance for red males and <0.5 dominance for blue males.

<table>
<thead>
<tr>
<th>Assemblage number</th>
<th>day 1</th>
<th>day 2</th>
<th>day 3</th>
<th>red</th>
<th>blue</th>
<th>more red than blue alpha males?</th>
<th>averaged U-value</th>
<th>mean SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>R</td>
<td>R</td>
<td>RR</td>
<td>1.3</td>
<td>0.0</td>
<td>yes</td>
<td>0.63 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>R</td>
<td>B</td>
<td>B</td>
<td>0.3</td>
<td>0.7</td>
<td>no</td>
<td>0.48 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>R</td>
<td>B</td>
<td>B</td>
<td>0.3</td>
<td>0.7</td>
<td>no</td>
<td>0.36 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>1.0</td>
<td>0.0</td>
<td>yes</td>
<td>0.50 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>0.0</td>
<td>1.0</td>
<td>no</td>
<td>0.19 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>RB</td>
<td>R</td>
<td>R</td>
<td>1.0</td>
<td>0.3</td>
<td>yes</td>
<td>0.63 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>0.0</td>
<td>1.0</td>
<td>no</td>
<td>0.33 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>1.0</td>
<td>0.0</td>
<td>yes</td>
<td>0.60 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>B</td>
<td>R</td>
<td>R</td>
<td>0.7</td>
<td>0.3</td>
<td>yes</td>
<td>0.62 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>R</td>
<td>RR</td>
<td>B</td>
<td>1.0</td>
<td>0.3</td>
<td>yes</td>
<td>0.63 ± 0.24</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>R</td>
<td>B</td>
<td>B</td>
<td>0.3</td>
<td>0.7</td>
<td>no</td>
<td>0.41 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>0.0</td>
<td>1.0</td>
<td>no</td>
<td>0.13 ± 0.06</td>
<td></td>
</tr>
</tbody>
</table>
Dominance of one morph over the other: all community members

The proportion of blue males ranking below red males in the dominance hierarchy of each mixed-color assemblage averaged over the three days (standardized U-value) is shown in table 2 (last column). The standardized U-value did not deviate from 0.50 (mean ± SE of the standardized U-value: 0.46 ± 0.05, RM-ANOVA, F_{1,22}=0.702, P=0.4). This suggests that in assemblages red and blue males did not systematically differ in dominance, in contrast to the dyadic combat situation in which red males had a higher chance to win than blue males (chapter 4).

We evaluated whether the dominance of red over blue is significantly different between a group and a dyadic situation by testing the standardized U-value against the probability that red wins against a blue opponent in dyadic combat (chapter 4). When weight-matched this probability was 0.85 (see figure 2 in chapter 4). The standardized U-value deviated highly significantly from this probability (RM-ANOVA: F_{1,22}=53.818, P<0.001). This means that the probability that red dominates blue is significantly lower in a mixed-color assemblage than in a dyadic combat.

Own-color bias in aggression

In mixed-color assemblages, males directed attacks to both color morphs. The proportion of attack behaviors directed to males of their own color relative to males of the other color did not differ between red and blue males (RM-ANOVA: F_{1,22}=0.003, P=1.0). Males of both colors biased their aggression towards males of their own color (Figure 1): the attack ratios deviated significantly from 3/7 (RM-ANOVA: blue males, F_{1,22}=5.384, P<0.05; red males RM-ANOVA: F_{1,22}=6.445, P<0.03).

Aggression level

The level of aggression expressed as the rate of received and the rate of performed attacks per individual is shown in figure 2. The aggression level in red assemblages was higher than in blue assemblages (RM-ANOVA: F_{1,12}=9.603, P<0.01, day effect: RM-ANOVA: F_{2,24}=11.988, P<0.001). The day effect is due to a lower aggression level on the first observation day: this temporal trend was also apparent in the results reported below.

Own-type biases in aggression would result in reduced aggression levels in mixed-color assemblages compared to single-color assemblages. In addition, the higher aggression level in red than in blue assemblages specifically predicts reduced aggression levels for red males in mixed-color assemblages compared to red assemblages. We therefore have a one-tailed alternative hypothesis for red males. We detected no difference in aggression level for blue males between mixed and blue assemblages (RM-ANOVAs, rate of received attacks: F_{1,17}=0.042, P=0.8, day effect: F_{2,34}=9.044, P<0.01; rate of performed attacks: F_{1,17}=0.627, P=0.4, day effect: F_{1,465,24,908}=6.323, P<0.05). By contrast, red males experienced a lower level
of aggression in mixed than in red assemblages (RM-ANOVAs, rate of received attacks: \( F_{1.17} = 3.145, P < 0.05 \), day effect: \( F_{1.395.23.718} = 8.945, P < 0.01 \); rate of performed attacks: \( F_{1,17} = 3.005, P = 0.05 \)).

**Number of territorial males**

Territorial males were defined as males that had their vertical bars switched on. The median and range of the number of territorial males per day, and based on averages over the three days, are listed for each treatment in table 3. The number of territorial males averaged over the three days did not differ systematically between treatments (ANOVA: \( F_{2,26} = 0.696, P = 0.5 \)). To test whether mixed-color assemblages allow more males to become territorial than single-color assemblages, we combined the data of red and blue assemblages. The median number and range of territorial males for single-color assemblages was: day 1: 3.0 (1-6); day 2: 2.5 (1-4); day 3: 2.5 (1-7); averaged: 2.7 (1-4). The number of territorial males averaged over the three days did not differ significantly between mixed- and single-color assemblages (independent t-test: \( t_{24} = -0.569, P = 0.6 \)).

Table 3 number of territorial males (vertical bars switched on) indicated for each treatment and day, and averaged over the three days. Shown are the median number of territorial males and the range per assemblage.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>day 1</th>
<th>day 2</th>
<th>day 3</th>
<th>averaged</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>range</td>
<td>Median</td>
<td>range</td>
</tr>
<tr>
<td>Blue</td>
<td>3</td>
<td>1-6</td>
<td>2</td>
<td>1-4</td>
</tr>
<tr>
<td>Red</td>
<td>3</td>
<td>1-4</td>
<td>3</td>
<td>2-4</td>
</tr>
<tr>
<td>Mixed</td>
<td>3</td>
<td>1-6</td>
<td>3</td>
<td>1-4</td>
</tr>
</tbody>
</table>

**Discussion**

We investigated aggression and social hierarchies among male Lake Victoria cichlids in assemblages composed of males of a single color morph and in such composed of males of two color morphs. We found that in mixed-color assemblages blue and red males did equally well in terms of social dominance. Even though in dyadic combat with blue males red males enjoy an advantage (chapter 4), neither the number of red alpha males relative to blue alpha males nor the dominance index score (standardized U-value) indicate dominance of males of one color over those of the other in mixed-color assemblages. A major difference between mixed-color assemblages and dyadic interactions is that in the latter case, red males not only experienced aggressive encounters with blue males, but also with other red males in which they lack the dominance advantage of red-blue fights. Reduced dominance of red over blue in a mixed group may arise from the energetic costs of
high levels of red-red aggression but also from experience of red males with other red males (which is absent if males are in pairs with a blue male). A similar result was obtained in a model on the self-reinforcing effects of dominance interactions in a group comprising of individuals of both sexes (Hemelrijk et al., 2003). Although males were initially dominant over females in the model, during sexual attraction when clusters of males were waiting in front of a female, these males interacted among themselves more often than otherwise. This reduced their dominance over females. Similar effects of self-organization may operate in territorial cichlid fish and the results illustrate the importance to validate in a group situation observations on dominance relationships made in isolated laboratory settings (see also Chase et al. 2002; 2003). The inability of males of either color morph to dominate those of the other color morph suggests that the red phenotype cannot displace the blue phenotype through male-male competition alone, despite the fact that red males enjoy an advantage in dyadic combat with blue males (chapter 4).

Males of both color morphs exhibited a bias in aggression towards males of their own color. We also found that there was more aggression in red assemblages than in blue assemblages. These results confirm aggression biases and levels measured in an isolated test situation, using simulated intruder choice tests (chapter 5; Dijkstra unpublished). Own-type biases in aggression predict that males in single-color assemblages experience a higher level of aggression than males in mixed-color assemblages. This prediction is particularly strong for red males, since there was more aggression in red than in blue assemblages. We found indeed that co-existence of males of both color morphs reduced the overall aggression level only for red males, whereas there was no difference for blue males. It is likely that for blue males the presence of the more aggressive red males outweighed the effect of reduced aggression in mixed-color assemblages. It follows that being in a mixed-color assemblage is more ‘beneficial’ for red males than for blue males: red males experience less costly male-male competition in the presence of blue males.

In Lake Victoria red populations of *Pundamilia* occur in the wild always sympatrically with at least one blue morph or species, whereas entirely blue *Pundamilia* populations are frequent (Seehausen & Van Alphen 1999; Seehausen & Schluter 2004). This is consistent with our finding that red males did not dominate blue males in mixed-color assemblages, as well as with the finding that co-occurrence of red with blue is beneficial for red males but not necessarily for blue males.

Own-type aggression biases might generate space for more territorial males in a mixed-color assemblage than in a single-color assemblage (Mikami et al. 2004). Our experiments did not support this prediction: the numbers of males that became territorial did not differ between single-color and mixed-color assemblages. Because of the small size of our experimental aquaria, our set-up may not have been a powerful test of this prediction though.

Theory and field observations suggest that frequency-dependent intrasexual selection on male nuptial coloration is important not just for co-
existence of ecologically similar species (Mikami et al. 2004), but also for the likelihood of sympatric speciation driven sexual selection by female mate choice (Seehausen & Schluter 2004; Van Doorn et al. 2004). Without such negative frequency-dependent selection there are three obstacles to sympatric speciation by sexual selection. First, the invasion of a novel male trait against the predominant female mating preference for the already established trait is problematic. Second, the stabilization of polymorphisms in trait and preference in the course of speciation is problematic, as is third, the stable co-existence of daughter species after speciation is completed (Arnegard & Kondrashov 2004; Van Doorn et al. 2004). Own-type biases in aggressive interactions between males generates the negative frequency-dependent selection required for facilitating invasion and stable co-existence of incipient species (Mikami et al. 2004; Seehausen & Schluter 2004; Van Doorn et al. 2004; chapter 2 & 3). Research on sympatric speciation by sexual selection has traditionally concentrated on the role of divergent female mate choice on male nuptial coloration. The possible role of male-male competition in the process has only recently been fully appreciated (Mikami et al. 2004; Seehausen & Schluter 2004; Van Doorn et al. 2004; this thesis).

This paper is a first attempt to test predictions derived from the male-male competition co-existence model in semi-natural assemblages of male cichlids. We showed (1) that despite dominance of red over blue males in dyadic contests, red and blue males do equally well in terms of aggression and social dominance in mixed-color assemblages, (2) that aggression is biased towards the own type in mixed-color assemblages, (3) that the aggression level was higher in red assemblages than in blue assemblages, (4) that co-existence in mixed-color assemblages reduced the level of aggression in red males. The results support some of the predictions derived from the male-male competition hypothesis, and suggest that male-male competition may be a significant force for species co-existence and diversification in species-rich cichlid fish communities.

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Territory quality affects female preference for male nuptial coloration in a Lake Victoria cichlid fish

Peter D. Dijkstra, Els M. van der Zee, Ton G. G. Groothuis
Abstract

The haplochromine cichlids of Lake Victoria represent a well-known test case for sympatric speciation driven by sexual selection. How a novel male color type can invade the population against the predominant female preference poses a major theoretical problem. Recent studies indicate that an advantage of a novel male color type in male-male competition for mating territories could promote their invasion. Such a competitive advantage may transmit to elevated fitness if territory quality can override the female preference for males of own color. We first showed in groups of *Pundamilia* males that the dominant male preferentially monopolized a large tube relative to a small tube, indicating the former are perceived as more valuable than the latter. The tubes mimic quality difference in a rocky crevice which serves as a focal point for male courtship display. In mate choice tests, *Pundamilia nyererei* females preferred conspecific over closely related *Pundamilia Pundamilia* males. These species differ strikingly in nuptial coloration, but little else. We then gave the heterospecific male a large tube and the conspecific male a small tube, or vice versa. Female preference for conspecifics in the control situation (both males small tube) was significantly diminished in favor of the heterospecific male when the latter had the large tube. Also, the female preference for conspecifics was lower when the heterospecific male had the large tube than when the conspecific male had the large tube. The results provide experimental evidence that territory quality can override the female preference for males of own color, lending support for the hypothesis that male-male competition can facilitate the establishment of a novel color.
Introduction

The colorful haplochromine cichlid species flocks of the Great African Lakes represents the most extreme examples of explosive speciation among vertebrates. The astounding species diversity in Lake Victoria (>500 species) likely evolved from a few ancestors in less than 15,000 years (Johnson et al. 2000; Seehausen et al. 2003). The promiscuous mating system of haplochromines, with exclusive female parental care in the form of mouthbrooding, is conducive to strong sexual selection by female mate choice. Evidence indeed suggests that many species have arisen in sympatry by the effects of sexual selection exerted by female choice on male color (Dominey 1984; Seehausen et al. 1997; Seehausen & Van Alphen 1999, Seehausen et al. 1999). This suggestion was inspired by the observation that many closely related sympatric species differ strikingly in male coloration but little else (Seehausen & Van Alphen 1999).

Recent theoretical investigations indicate sympatric speciation by sexual selection requires unrealistically stringent conditions (Arnegard & Kondrashov 2004; Kirkpatrick & Nuismer 2004; Van Doorn et al. 2004). In particular, it is unclear how a novel male phenotype can invade without frequency-dependent natural selection. The stable co-existence of incipient species is likewise problematic. Van Doorn et al. (2004), Seehausen & Schluter (2004) and Mikami et al. (2004) independently proposed that male-male competition for mating territories could generate this frequency-dependent selection on male nuptial coloration. Haplochromine males establish individual territories on leks, and females visit these assemblies for egg fertilization. Territory ownership is a prerequisite to gain access to spawnings (Parker & Kornfield 1996; Maan et al. 2004). Hence, competition over territory sites is intense, and is likely to affect sexual selection (Seehausen & Schluter 2004). Moreover, nuptial coloration in haplochromines is not only important in mate choice (Seehausen & Van Alphen 1998), but also in aggressive interactions (chapter 2 & 4). Van Doorn et al. (2004) showed in simulations that one factor facilitating sympatric speciation by sexual selection is if territorial males bias aggression towards phenotypically similar rivals. Such a bias in aggression is consistent with the fact that closely related cichlid species when at the same locality in Lake Victoria varied more in male nuptial color than expected by chance (Seehausen & Schluter 2004). Territorial males also tended to have territorial neighbors of species with different nuptial coloration from themselves (Seehausen & Schluter 2004).

This proposed advantage for rare male color types may not easily translate into a higher reproductive success, unless it positively affects female choice against the predominant female color preference. This might be the case if rare male color types are able to obtain or construct better quality territories in terms of surface area and/or number and size of rocks and crevices, and if territory quality can override the female preference for color (Seehausen & Schluter 2004).
Several studies on lekking animal species have explored the role of male and territory characteristics in mate choice (see Höglund & Alatalo 1995 for review). Few have experimentally tried to disentangle the effects of male and territory characteristics (Borgia 1985a; Nelson 1995; Madden 2002). This is also true for cichlids (Nelson 1995), although evidence suggests territory quality and intrinsic male characteristics, such as nuptial dress co-determine mate choice in lekking cichlids (McKay et al. 1990; McKay 1991; Taylor et al. 1998; Kellog et al. 2000; Maan et al. 2004). For example, males of several sand-dwelling Lake Malawi cichlids construct and defend sand structures (termed bowers) that function mainly as display sites (McKay et al. 1990). Field studies indicated bower shape and size (McKay 1990; Taylor et al. 1998; Kellog et al., 2000) as well as central bower position within the lek (McKay 1991) correlated with elevated male mating success. Some of these authors (Taylor et al. 1998) suggest these display sites are ornaments indicating the physical or genetic quality of the male (Zahavi 1975). This has also been suggested for bower constructions of bowerbirds (Borgia et al. 1985; Borgia 1985a; Borgia 1985b). There appear to be no published studies on the relative importance of male and territory characteristics in mate choice in the context of sympatric speciation.

Pundamilia nyererei (Witte-Maas & Witte 1985) and Pundamilia Pundamilia (Seehausen et al. 1998a) are very closely related rock-dwelling sibling cichlid species. They occur sympatrically at many locations in Lake Victoria, and represent a common color polymorphism in haplochromines (Seehausen 1997; Seehausen 1996; Konings 2001; Smith & Kornfield 2002). Females are cryptically colored. Males of P. nyererei are bright red with yellow flanks and P. Pundamilia males are metallic blue. We will also refer to Pundamilia nyererei as ‘red’ and to P. Pundamilia as ‘blue’. At Kissenda Island, P. nyererei and P. Pundamilia mainly occur as distinct phenotypes, with occasional intermediate phenotypes, indicating some hybridization. P. nyererei and P. Pundamilia from Kissenda Island are genetically very similar species (Seehausen unpublished), making it a suitable system to measure mate choice based on color. Females of both species preferred conspecific males in mate choice experiments when colors are visible, but not when they are masked (Seehausen & Van Alphen 1998). Pundamilia males establish individual territories over rocky and sandy bottoms, often with a rocky crevice as the focal point of their territory. Males compete for territories that vary in size, number and shape of rocks and interstitial crevices, and number of neighboring territorial males (Maan et al. 2006c). In a field study, P. nyererei female choice was positively influenced by territory size (Maan et al. 2004).

In the laboratory study described here we tested whether territory quality affects mate choice and can override the color preference of P. nyererei females. To create a difference in territory quality, we used PVC tubes of two sizes. The tubes mimic a rocky crevice serving as a spawning location in a male’s territory. We predicted that a large tube mimicking a large cave corresponds to a relatively ‘high-quality territory’. This was based on the observation that in the presence of large
and small tubes, dominant males often occupy the large tube. To test our assumption that dominant males prefer a large tube, we investigated dominance hierarchy formation and tube occupation among groups of _Pundamilia_ males. To confirm an earlier finding that females of _P. nyererei_ have a color preference for conspecifics when both males have similar territories (Seehausen & Van Alphen 1998), we allowed females in simultaneous mate choice tests to choose between a conspecific mate and a heterospecific one (_P. Pundamilia_) both occupying a small tube (same-tube treatment). Finally, we repeated the choice tests, but gave the blue male a large tube and the red male a small tube (blue-large-tube treatment) or vice versa (red-large-tube treatment). We predicted that the female preference for conspecifics is reduced in the blue-large-tube treatment relative the same-tube and the red-large-tube treatment.

**General Methods**

*Fish and general housing condition*

All fish originated from the Mwanza Gulf in Lake Victoria, Tanzania. We used wild-caught individuals and first generation laboratory bred offspring. Laboratory breeding was carried out in single-species breeding tanks. All fish used in this study were sexually mature (standard length > 50mm). Haplochromine males defend individual territories over rocky/sandy bottoms, and court passing females. Courtship resembles that of other haplochromine species (McElroy and Kornfield, 1990). It typically starts with an interested male approaching the female, immediately followed by lateral display, in which the male positions itself perpendicular to the female and spreads all fins. This is followed by quiver, a high-frequency shaking movement of the body. Finally, the male leads the female to the centre of the territory with exaggerated tail beats (lead swim), where it presents the egg dummies on his anal fin. All these courtship behavioral elements can also occur independently, or in a different sequence. Mating occurs in rocky crevices, and immediately after spawning the female leaves the territory with the eggs in her mouth (Seehausen 1996).

We fed all fish flake food (TetraMin Tropical Fish Flakes) twice a day and a mixture of ground peas and shrimp with added vitamins twice a week. The test fish were fed after testing. All aquaria were connected to a central biological filtration system and water circulated continuously. Water temperature was kept at 25 ± 2°C and a 12:12 h light:dark cycle was maintained. All aquaria contained a substrate of gravel.

**Experiment I: Tube occupation**

In this experiment we tested whether dominant males prefer to occupy a large or a small tube.
Methods

Fish and housing

For this experiment we used males of several *Pundamilia* species to obtain a decent sample size. Specifically, we used *P. nyererei* and *P. Pundamilia* males and females from Kissenda Island, supplemented with *P. Pundamilia*-like males from Luanso Island. At Luanso Island there is only a single *Pundamilia* species, consisting mainly of individuals that resemble *P. Pundamilia*, but also of a few that resemble *P. nyererei* (Seehausen 1996; 1997). Specifically, in terms of males, we used 38 *P. Pundamilia* first generation (standard length range 66 mm – 85 mm), ten *P. nyererei* first generation (67 mm – 84 mm), 14 *P. nyererei* wild-caught (69 mm – 102 mm) and eight *P. Pundamilia* wild-caught (104 mm – 114 mm). All these males originated from Kissenda Island (Seehausen 1996). In addition, we used eight first generation *P. Pundamilia*-like males from Luanso Island (57 mm – 65 mm). Prior to use in a test, males were housed individually with visual access to a *P. nyererei*, *P. Pundamilia* or *P. Pundamilia*-like male behind a transparent sheet for at least three days. This housing condition encouraged territoriality and avoided unwanted effects of social deprivation. We used three test tanks (one tank of 150 x 40 x 50 cm and two of 250 x 46 x 66 cm, l x w x h). In each test tank we permanently housed 8 *P. nyererei* first generation females.

Procedure

To test our assumption that dominant males preferentially occupy the large tube we allowed dominance hierarchy formation among males and analyzed tube occupation by the most dominant male in a community. The test tanks were visually divided into three equally spaced compartments with white tape on the front side of a test tank. In one end compartment we horizontally placed a large dark grey tube on the bottom (⌀ 15 cm, length 21 cm) to create a large territory, and in the other end compartment a small dark grey tube (⌀ 5 cm, length 7 cm) corresponding to a small territory. One day prior to testing, a group of eight *Pundamilia* males were allowed to acclimatize to the tank, where eight *P. nyererei* females were already established. A group consisted of either solely *P. nyererei* (N=5 groups), *P. Pundamilia* (N=8 groups), or *P. Pundamilia*-like males (N=1 group). Males of different species were never put together in a single group.

The fish were confined in the midsection with opaque sheets without tubes. The presence of females ensured courtship behavior of the males during the actual test. We used a total of 78 males. Since only the most dominant male was of interest, 34 subdominant males were used a second time in another test with a different group composition of males; the two most dominant males in each group were used only once. The interval between use of the males was at least three days. Prior to a test, we individually clipped the males on their dorsal and caudal fin, using scissors, for individual recognition. The fin clips healed within a week. On a
test day, we removed the opaque sheets. Males were then allowed to form a hierarchy for one hour, before we started an observation (for behavioral observations to assess hierarchies see below). After an observation, we immediately returned males to their individual compartment.

**Behavioral observations**

We observed a group of fish for 30 minutes (two cases) or 60 minutes (12 cases). The males showed the common repertoire of aggressive and sexual behaviors in cichlids (Baerends and Baerends-Van Roon 1950). We recorded aggressive behaviors, consisting of either chases, defined as a male that charges towards another male, or aggressive display, defined as a male that erects his fins (Baerends and Baerends-Van Roon, 1950). For each behavior we noted in which compartment it took place, or from which compartment a behavior was initiated in case a male moved over compartments. In each aggressive interaction we were in most cases able to assign a winner and a loser. This information is used to derive a dominance hierarchy (see below). Aggressive interactions without a winner and a loser were not recorded. In addition, we recorded in which compartment male courtship took place, defined as either lateral display, quiver, lead swim or presentation of egg-dummies (Seehausen 1996), but we did not distinguish between these different types of courtship display. No spawning took place during the experiments. All observations were conducted by two persons, one person acted as the observer, and the other noted all observations on a sheet. The size of the group and the frequency of interactions were such that it was possible to record all individual interactions.

**Analysis**

To determine the most dominant male (*alpha* male) in the tank, we generated dominance matrices for each group. To derive a dominance hierarchy, we used the average Dominance Index, which is the average of the relative dominance (RD) index of all pairs in which the individual interacts. This measure of dominance is recommended by Hemelrijk et al. (2005). The RD index of each pair is calculated as the number of times an individual won from a certain opponent divided by the total number of fights in which the pair was involved with each other. The average Dominance Index of an individual is the average of all its RD indices with all its interaction partners. The fish with the highest average Dominance Index was defined the *alpha* male. The average Dominance Index was a reliable measure to determine the *alpha* male; in all communities the *alpha* male was most dominant in terms of number of subordinate community members. There were no cases of intransitivities related to the male with the highest average Dominance Index.

To evaluate tube occupation by the *alpha* male, we compared the percentage of aggressive behaviors and courtship events in or initiated from the compartment with the large tube relative to the one with the small tube using Wilcoxon signed-ranks (WSR) tests.
Results

To analyze which tube the alpha male prefer to occupy, as measured by the location of attacks and courtship events, we first determined which the alpha male was in each community. In all communities (N=14) we were able to identify such a male.

The frequency of aggressive behaviors of the alpha male was 3.0/min (1.9-5.1). Alpha males initiated more attacks to other males from the section with the large tube relative to the section with the small tube (Figure 1A, WSR test: Z=-2.229, P=0.026, N=14). In all 14 communities courtship took place. The frequency of courtship behaviors of the alpha male was 0.08/min per tank (0.03-0.23). Alpha males performed significantly more courtship behavior in the large-tube compartment relative to the small-tube compartment (Figure 1B, WSR test: Z=-2.313, P=0.021, N=13, one community without courtship of the alpha male was excluded). These results indicate that alpha males have a preference for the large tube.
Experiment II: Mate choice

In this experiment we tested whether tube size can override the female preference for conspecifics using mate choice tests.

Method

Same-tube treatment

Fish and housing condition

We used as test fish first generation females (standard length range=50-73 mm) of *P. nyererei*, bred from fish originating from Kissenda Island (Seehausen 1996). At this location, *P. nyererei* (red) and *P. Pundamilia* (blue) defend neighboring territories (Seehausen 1997). First generation males and females were raised in mixed-sex mixed-species groups. At least four weeks prior to testing, 38 females were housed in all-female tanks in groups of circa 10 individuals. We selected gravid females for testing. Gravidity was determined by swelling of abdomen and urogenital opening (Seehausen & Van Alphen 1998). After testing females were put in other all-female tanks. We assembled male stimulus pairs from 17 *P. nyererei* males, of which 14 were first generation and three wild-caught (77-103 mm), and from 18 *P. Pundamilia* males, of which 14 were first generation and four wild-caught (77-102 mm). The same males were used as in experiment I. Prior to use in a test, males were individually housed in the same way as in experiment I for at least a week.

Procedure

To test the female preference of red females for conspecific mates, we carried out simultaneous mate choice tests in which we allowed females to choose between a blue and a red male. The test tank (150 x 40 x 50 cm) was divided into three equally spaced compartments by transparent Perspex sheets. In both male compartments (at the sides of the tank) we placed a small dark grey PVC tube, similar to the one used in experiment I. Tubes were placed horizontally on the bottom. Since tube size was kept identical between males, we refer to this as 'same-tube treatment'. The left and right position of the stimulus male with respect to species was randomized across tests. Stimulus males were matched for standard length, and the difference was less than 5%, measured as \((100x(L-S))/S\), where L= the longest and S= the shortest fish.

For each trial, we placed a stimulus male in each of the side compartments. They were left overnight in the experimental tank. A red male was placed in the central compartment to maintain territoriality in both stimulus males. On a test day, the red male in the central compartment was removed. After we fitted opaque sheets on either side of the central compartment, we gently put in the test female.
The opaque sheets prevented the fish from seeing each other. The female and males were allowed to settle for one and a half hour. Females were given a small tube as shelter. At the start of the pre-test, we removed the opaque sheets. Females were contained in the central compartment by a double layer of transparent sheets: one without holes and one with circular holes ($\odot 2.3$ cm). The holes were small enough for females but not males to swim through. During this period, the females were allowed to see both males and their territories for 5 minutes. To begin a test, the transparent Perspex sheets without perforations were removed, leaving the transparent perforated Perspex sheets. The actual test lasted 25 minutes. We videotaped the behavior of the female and the males. From the tapes, we recorded male courtship behaviors (lateral display, quiver, lead swim and presentation of egg-dummies). We recorded the frequency with which a male display was followed by a female approach (positive female response). For a reliable measure of female preference, both males need to display several times to a female. Therefore, we defined a successful trial when (1) both males courted at least four times while the female was visiting his compartment and (2) when females showed at least one positive response to at least one of the males (Seehausen & Van Alphen 1998). We ran a total of 14 successful trials. Most stimulus males were used only once. Some males were used twice (red males $N=3$, blue males $N=3$). When using a male for the second time, it was never paired with the same male (Kodric-Brown & Johnson 2002; Pauers et al. 2004).

**Red-large-tube and blue-large-tube treatment**

In this experiment we used the same stimulus males and selected females from the same 38 individuals as in the same-tube treatment. To test whether females prefer males that occupy a ‘high-quality’ territory, and to test whether this can override color-based female mate choice, we carried out mate choice tests with an identical set-up as described above, but with the following adaptations. In the male compartments we created a difference in territory quality in a similar fashion as in experiment I: one male was given a small tube and the other male a large tube. Females were exposed to two treatments: one in which the red male occupied the large tube and the blue male a small tube (referred to as ‘red-large-tube treatment’), and one in which the blue male received the large tube and the red male a small tube (referred to as ‘blue-large-tube treatment’). We ran a total of 14 successful trials in each treatment. Some males and females acted in both treatments. Between use in the different treatments, females were housed in all-female tanks, with an interval of at least one day. Although individual identity of the females was unknown, we ensured that the same female could act no more than once in each of the two treatments. Like in the same tube treatment, we used some males twice in one treatment (red-large-tube: red males $N=3$, blue males $N=2$; blue-large-tube: red males $N=5$, blue males $N=5$). When using a male for the second time, it was never paired with the same male (Kodric-Brown & Johnson 2002; Pauers et al. 2004).
Analysis

The proportion of male courtship displays resulting in an approaching female relative to the total number of male displays is our measure of mate choice. It controls for possible differences in display behavior between males. We refer to this measure of mate choice as ‘proportion of positive female response’.

The frequency of male courtship display was calculated as the number of behaviors relative to the time a female spent in his compartment. Prior to a test females were allowed to see both males and their territories for 5 minutes; during this period males did not differ in courtship frequency (WSR tests: Zs>-1.475, Ps>0.14, N=14 for same-tube and both red-large-tube and blue-large-tube treatment).

To test whether females prefer one type of male, the proportion of female positive response to each male within trials was compared with WSR tests. The ‘female preference for conspecific male’ was calculated as the proportion of positive female response to the red male (R) minus that to the blue male (B) divided by the sum of proportion of positive female response (R-B/(R+B). The denominator corrects for the absolute proportion of positive female response. We compared female preference for conspecific male between the three treatments with a Kruskal-Wallis test. Post-hoc testing between treatments was done with Mann-Whitney U (MWU) tests.

The data in the text are presented as median (25th - 75th quartiles). Analyses were carried out with SPSS 12.0.1. All quoted probabilities are for two-tailed tests of significance.

Results

Same-tube treatment

Figure 2 (left data point) shows the female preference for conspecific male. Females had a significant preference for conspecifics: the proportion of positive female response was significantly higher to conspecific than to heterospecific males (Figure 2; table 1).

The frequency of male display for red males was 0.10 (0.04-0.27) and for blue males 0.15 (0.05-0.27). This was not different between species (WSR test: Z=-0.157, P=0.875, N=14).

Red-large-tube and blue-large-tube treatment

Figure 2 shows the female preference for conspecific males in the blue-large-tube treatment and the red-large-tube treatment. In the red-large-tube treatment females had a significant preference for conspecific male, whereas in the blue-large-tube treatment this preference was reduced towards a nonsignificant preference for the heterospecific male (Figure 2; table 1).
The frequency of male display did not differ between red and blue males neither in the red-large-tube nor in the blue-large-tube treatment (frequency of male display, blue-large-tube treatment: red males 0.08 (0.06-0.16), blue males 0.08 (0.06-0.14), WSR test: \( Z = -1.099, P = 0.272, N=14 \); red-large-tube treatment: red males 0.07 (0.03-0.11), blue males 0.11 (0.05-0.16), WSR test: \( Z = -1.350, P = 0.177, N=14 \)). This indicates that tube size did not affect the frequency of male display and thereby female choice.

**Comparison between treatments**

We tested the prediction that the preference for conspecific male can be reduced by adding a large tube in the territory of a heterospecific male. This prediction was supported. The female preference for conspecific male was different between treatments (Kruskal-Wallis test: \( \chi^2 = 7.39, df = 2, P = 0.025 \)), which was due to females having reduced female preference for conspecific male in the blue-large-tube treatment: the female preference for the conspecific male was significantly lower in the blue-large-tube treatment compared to the red-large-tube treatment.
(Figure 2, MWU test: $U=49$, $P=0.024$, $N_1=14$, $N_2=14$) as well as compared to the same-tube treatment (Figure 2, MWU test: $U=46$, $P=0.016$, $N_1=14$, $N_2=14$). This indicates that tube size can override the female preference for males of own color.

The frequency of male display did not differ between treatments for red males (MWU test: same-tube versus red-large-tube treatment, $U=92$, $P=0.76$; same-tube versus blue-large-tube treatment, $U=95$, $P=0.89$) nor for blue males (MWU test: same-tube versus blue-large-tube treatment, $U=98$, $P=0.98$; same-tube versus red-large-tube treatment, $U=93$, $P=0.82$).

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Red male Median (25th - 75th quartiles)</th>
<th>Blue male Median (25th - 75th quartiles)</th>
<th>Z</th>
<th>P</th>
</tr>
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<tbody>
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<td>Same-tube $N=14$</td>
<td>0.22 (0.16-0.34)</td>
<td>0.16 (0.00-0.20)</td>
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<td>0.004</td>
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<td>Blue-large-tube $N=14$</td>
<td>0.07 (0-0.18)</td>
<td>0.10 (0.07-0.20)</td>
<td>-0.785</td>
<td>0.433</td>
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<td>Red-large-tube $N=14$</td>
<td>0.19 (0.13-0.24)</td>
<td>0.09 (0.05-0.15)</td>
<td>-2.354</td>
<td>0.019</td>
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</tbody>
</table>

### General Discussion

The question addressed here is whether territory quality influences mate choice and can override the expression of female preference for conspecific male nuptial coloration in *P. nyererei*. Such an effect can facilitate the invasion of novel male phenotypes in case these have a competitive advantage over the ancestral morph. The results are consistent with our expectations. We first confirmed our assumption that males preferentially occupy a large tube relative to a small one. This finding indicates that males are sensitive to quality cues of a territory. *Alpha* males performed more courtship displays in the vicinity of the large tube than near the small tube. In addition, *alpha* males preferentially charged rival males from the compartment with the large tube. In mate choice experiments, we subsequently showed that females prefer males of their own color when territory quality is kept similar, confirming earlier results (Seehausen & Van Alphen 1998). This preference was changed in favor of the heterospecific male when that male had a large tube. Also, the female preference for conspecific male was significantly lower in the blue-large-tube treatment than in the red-large-tube treatment. Thus, tube size can alter and override the female preference for conspecific male. The effect of territory
quality may thus contribute to the establishment of rare color types if those have an advantage in intrasexual competition.

Several experimental studies demonstrate the relative importance of male and territory characteristics in mate choice. Most of these concern animals with male brood care (Alatalo et al. 1986; Jones & Reynolds 1999; Östlund-Nilsson 2000; Calsbeek & Sinervo 2002; Brouwer & Komdeur 2004). Few studies have experimentally tested this in lekking animal species (Borgia 1985a; Nelson 1995; Madden 2002). *Oreochromis mossambicus* females spawned more often in large sand pits than in small sand pits in the absence of males (Nelson 1995). Our findings are congruent with Nelson’s results as well as with a number of correlative studies on lekking cichlids (e.g. Kellogg 2000; McKay et al. 1990; Maan et al. 2004).

Rock-dwelling cichlids form lek aggregations where multiple males occupy individual territories over rocky or sandy sites (Seehausen 1996; Maan et al. 2004). The size of their territories, as well as the size and number of rocky structures contained in them can vary among territorial males. In our experiment we aimed at manipulating crevice size by using PVC tubes. Why do females find males occupying large tubes, or a high-quality territory more attractive? First, females may gain indirect benefits by mating with males with a high-quality territory, because such territories may be more costly to obtain. Territories may then function as ‘ornaments’ that can honestly signal superior genetic or phenotypic quality of the owner (Zahavi 1975). Such a ‘good-genes’ scenario is especially likely in species without paternal care. The fact that dominance is related to occupation of the large tube relative to the small one is consistent with this ‘good genes’ interpretation. Several studies have indicated that dominance is related with heritable fitness (Moore 1990; Alatalo et al. 1991). Secondly, there are potential direct benefits from large crevices since they may provide better shelter during the courtship and spawning event (Kellogg et al. 2000; Svensson & Kvarnemo 2003).

In mate choice decisions the effect of mate quality (for example body size) may oppose species recognition cues (Pfennig 1998 and references herein). Our study is intriguing because this conflict is imposed by territory quality.

It seems unlikely that the female preference for the male with the large tube was mediated via the male’s courtship activity. Males readily accepted both types of artificial caves as the focal point of their territory. In addition, vertical coloration bars, which are an indication of territoriality (Baerends & Baerends-Van Roon 1950; Maan et al. 2004), were in all cases maximally expressed in both stimulus males. Furthermore, we could not detect differences in male courtship effort associated with the size of the tube which might have been instrumental in determining female preference (Haesler & Seehausen 2005). In any case, our results show that territory quality affects mate choice.

Traditionally, the explosive speciation of Lake Victoria cichlid fish has been explained by sexual selection exerted by female mate choice for male nuptial coloration (Seehausen et al. 1999a; Kocher 2004). The importance of male-male
competition in sexual selection and rapid sympatric speciation in Lake Victoria cichlids has recently been fully acknowledged (Mikami et al. 2004; Seehausen & Schluter 2004; Van Doorn et al. 2004; this thesis). Our findings give further credence to the theory that male-male competition for mating territories may facilitate the invasion of novel color types, and possibly sympatric speciation by disruptive sexual selection.

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Social stimulation, nuptial coloration, androgens and immunocompetence in a sexual dimorphic cichlid fish

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Abstract
The nature of the costs maintaining honesty of sexual signaling in inter- and intrasexual interactions remains a contentious issue. For carotenoid-based color ornaments, it has been hypothesized that the honesty of the signal is enforced when carotenoid allocation to color expression is traded off against carotenoid availability for immunocompetence. In addition, honesty is enforced if androgens required for color expression are immunosuppressive. We tested whether there is a trade-off between color expression and immunocompetence in a lek-breeding haplochromine cichlid fish from Lake Victoria with a carotenoid-based nuptial dress, *Pundamilia nyererei*. First, we showed that expression of red nuptial coloration and social rank in a group were positively correlated. We then successfully manipulated the level of color advertisement by socially stimulating individually housed males with a rival male: stimulated males developed larger areas of red nuptial coloration and had higher levels of circulating 11-ketotestosterone than non-stimulated males. We subsequently tested the humoral immune response to a novel antigen (sheep red blood cells). There was no overall significant effect of social stimulation on antibody production, but in the stimulated males the degree of red coloration and the antibody response were negatively correlated. This significant interaction between color and treatment thus shows that the negative correlation between coloration and antibody production is dependent on the (manipulated) social environment. This study provides correlational and experimental evidence for a trade-off between expression of a sexual trait and one component of immune function in fish. We found no evidence for a direct effect of 11-KT on antibody production, and we discuss alternative mechanisms that could mediate the trade-off.
Introduction

In many animals males advertise extravagant characteristics that are used as cues in both female mate choice and male-male competition (Zahavi 1975; Andersson 1994). In female mate choice, females are expected to prefer males with traits that are honest indicators of quality that are passed on to their offspring (Hamilton & Zuk 1982; Johnstone 1995). Such traits may also signal social status and resource holding potential in intrasexual communication (Parker 1974), indicating dominance and/or fighting ability, thereby preventing or reducing the costs of combats with a predictable outcome (Rohwer 1975; Maynard Smith & Harper 1988; Berglund et al. 1996). For example, a male can increase color expression to indicate superior quality, thereby deterring rivals. Alternatively, a male can reduce the level of signaling to avoid conflict with more powerful opponents (Candolin 1999). If signaling is costly due to male-male competition and the costs decrease with an increase in the quality of the male, frequent testing through male-male competition could ensure the honesty of status-signaling (Rohwer 1975; Maynard Smith & Harper 1988). However, theory suggests that social control is not enough to maintain honest signaling and that contest-independent costs are needed to prevent the occurrence of cheating in a population (Johnstone & Norris 1993).

It has been suggested that honesty of signaling male quality is ensured if sexual signaling is compromised by immunocompetence (Hamilton & Zuk 1982). This trade-off between signaling and immune function has been extensively investigated in the context of female mate choice for high quality males (i.e. Roberts et al. 2004), but it may also be relevant in ensuring honesty in intrasexual signaling (Johnstone & Norris 1993; Buchanan et al. 2003). This is particularly important in species with lekking behavior where male contest competition over mating territories is severe, and likely to be a primary component of sexual selection. In general, success in contest is probably associated with male condition and overall health (Borgia 1979; see also Freeland 1981; Koskimäki et al. 2004), and reduced signaling as a result of infection or immune activation has been demonstrated in several animal species (Milinski & Bakker 1990; Houde & Torio 1992; Faivre et al. 2003; Brawner et al. 2000).

Two direct trade-offs between immune function and sexual signals have been proposed: competition for carotenoids (Lozano 1994; von Schantz et al. 1999) and the dual effect of androgens stimulating sexual traits and suppressing immune function (Folstad & Karter 1992). Red, orange and yellow coloration are often carotenoid-dependent ornaments (Hill 1999). Carotenoids need to be ingested from food and are considered a limiting source in nature (e.g. Grether et al. 1999). They are also required for an effective immune system (Lozano 1994). Competition for carotenoids between ornaments and immune function may hence enforce honesty on carotenoid-based sexual signals (Lozano 1994; von Schantz et al. 1999). In accordance with this hypothesis, studies in several species have shown that carotenoid supplementation can enhance ornament coloration (e.g. Kodric-Brown
1989; Evan & Norris 1996; Hill 1992; Blount et al. 2003, but see Fenoglio et al. 2002), as well as immunocompetence (for review see Bendich 1993; Christiansen et al. 1995; Blount et al. 2003; McGraw & Ardia 2003; Grether et al. 2003; but see Navarra & Hill 2003).

Folstad & Karter (1992) proposed that display might reflect an individual's immunocompetence, because testosterone is responsible for the development of exaggerated sexual signals (Wingfield et al. 1990) but may also have detrimental effects on the immune system. Since immunosuppression leads to higher susceptibility to disease, only high quality males could afford to display sexual characteristics fully. Studies on the immunocompetence handicap hypothesis have yielded ambiguous, if not contradictory results (Roberts et al. 2004). This may be due to our lack of understanding of the mechanisms by which testosterone modulates immune function (e.g. Marsh 1996).

Few studies on the trade-off between sexual signaling and immune function concern fish. Haplochromine cichlids exhibit a pronounced sexual dimorphism, with often drab females and brilliantly colored males (Seehausen 2000). They are maternal mouth brooders, and maternal care continues for up to several weeks after the free-swimming juveniles are released. For males territory ownership is a prerequisite to gain access to spawnings (Parker & Kornfield 1996; Maan et al. 2004). Male contest competition over spawning territories is intense. The complete lack of paternal care, and intense territoriality leads to strong inter- and intrasexual selective pressure on male color patterns (Maan et al. 2004; Seehausen & Schluter 2004; this thesis).

In this study we focus on *Pundamilia nyererei* (Witte-Maas and Witte, 1985) a Lake Victoria cichlid fish with a carotenoid-based nuptial dress (Maan et al. 2006c). We first established whether coloration is a signal of social dominance (experiment I). Then we asked whether sexual advertisement is stimulated in a male-male competition context (Experiment II). In the same experiment, we analyzed whether honesty of signaling may be enforced by an immunological cost, and if so, whether this trade-off is caused by androgens.

**Material and Methods**

**Fish**

We studied first generation offspring of wild-caught *Pundamilia nyererei* from Makobe Island in the Speke Gulf, Lake Victoria, Tanzania. Males of *P. nyererei* are crimson dorsally, yellow on their flanks, and have a crimson dorsal fin. We used 31 adult fish (weight, mean ± SE=16.6 ± 1.1, standard length [SL] mean ± SE=83.5 ± 1.5). The animals were raised at the Department of Animal Ecology, University of Leiden (The Netherlands). They were transported to the Zoological Laboratory in Haren, The Netherlands, two months before commencement of the experiments.
Housing

All males were housed in a large stock aquarium (300L) without females at least two months before commencement of the experiments. Thereafter, they were kept individually in 15L (Experiment I) or 50L compartments (Experiment II) containing a PVC tube as a hiding place. The sides and the back of the aquaria were covered with black plastic. All aquaria were connected to a central biological filter system and water circulated continuously. Water temperature was kept at 25 ± 2°C and a 12:12 h light:dark cycle was maintained. All aquaria contained a gravel substrate. The fish were fed flake food (TetraMin Tropical Fish Flakes) six times per week and a mixture of ground shrimps and peas twice per week.

Experiment I

Males were housed in isolation for at least one week in 15L compartments to minimize the effects of prior experience. The experiment started when a group of males, consisting of 8 individuals, were released in large aquaria (760L). Each aquarium contained 10 *Melanotaenia lacustris* as background fish. These fish lack bright coloration, are non-territorial, and are standard background fish in our laboratory to reduce the level of aggression. Each male in a group, individually recognizable by a fin clip, was followed on the fourth day following release by focal sampling for 5 minutes. The aggression varied from displays to charges (see Baerends & Baerends-Van Roon 1950 for more details of aggressive interactions in cichlids). We scored the frequency of chases and displacements. A chase was defined as charging another fish. A displacement was defined as a fish that was displaying, followed by his opponent fleeing, or when two fish were displaying to each other, followed by one of them fleeing. In each aggressive interaction we were in most cases able to identify a winner and a loser (the one that is fleeing after a chase or display). Thus, during focal sampling of an individual, we gathered information on which male the focal male defeated as well as which male defeated the focal male. We used the number of won interactions (sum of chases and displacements) to rank the fish in each group from dominant to subordinate.

After focal observation of a male, two judges independently scored the intensity of the red nuptial coloration on a 0-5 point scale, with a score of 5 representing the brightest red male. There was general agreement between judges: the red score did not differ between judges (paired t-test: $t_{31}=-1.72$, $P=0.1$), and the scores of the two judges correlated well (Cohen’s Kappa: 0.64, $P<0.001$, $N=32$). Color measurements by means of photography as described below for experiment II was not done, since moving individual fish from a group for photography would potentially lead to rapid changes in hierarchy and red scores. We observed 4 groups of males.
Statistics

The relationship between rank (fish with the largest number of won interactions rank 1, fish with the smallest number of won interactions rank 8) and red score (averaged over the two judges) was calculated by the Spearman rank correlation for each group separately. We used Fisher’s Combination Test to combine significance levels over several samples (Sokal & Rohlf 1995).

Experiment II

Experimental design

At the start of the experiment, all males were housed individually and divided into two treatment groups: one group in which males were socially stimulated with a similar-sized neighboring conspecific rival male in the same aquarium (N=16, weight mean ± SE=17.2 ± 1.7, SL mean ± SE=84.5 ± 2.3), and one group in which males were not stimulated (N=15, weight mean ± SE=16.0 ± 1.5, SL mean ± SE=82.4 ± 2). The non-stimulated males were given a small juvenile conspecific neighbor (SL<1cm) to avoid unwanted effects of social isolation. Within aquaria, fish were separated from one another by transparent PVC sheets (thickness 1 mm).

After housing the males in the two treatments (day 0) we followed the development of nuptial coloration by photographing males on 7 occasions (day 0, 4, 6, 10, 17, 20, 25). On day 14 all males were challenged with sheep red blood cells (SRBC, see below for details). 14 Days later (on day 28), around the time of the peak of the primary response (P. D. Dijkstra, W. Pardijs and T.G.G. Groothuis unpublished data), we took a post-immunization blood sample. The primary response is the antibodies produced upon first exposure to an antigen. Weight measurements were taken on day 0, 10 and 25.

Color measurement

We photographed all males under standard conditions always before weighing. Individual male fish were briefly confined in a Perspex cuvette with water. Fish were kept in place by gently squeezing the male between a grey PVC sheet and the front window. One flank of a male was photographed, and the left or right flank was randomly chosen. The distance between the camera lens and front window was 20 cm. We used a digital photo camera (Sony Cybershot DSC-F717) with flash lights. Both flash lights were located 40 cm to the left and right from the camera. We analyzed the colors of the fish body, excluding the fins and the eyes, in Sigmascan Pro 4.0 (SPSS Inc.). For color analysis, we followed the procedure of Maan et al. (2004; 2006c) described for color analysis in our study species P. nyererei. To determine the area of red, we defined criteria by a combination of hue and intensity (hue 0–26 plus 232–255, saturation 40–97%). The resulting area of red was expressed relative to the total body area, yielding a percentage of red body coverage.
that fulfilled these criteria (Maan et al. 2004; 2006c). This method corresponds well to the color score judged by eye on a scale of 0-5

**Haemagglutination assay**

Males were immunized with sheep red blood cells (Harlan Inc., The Netherlands). The cells were washed three times with phosphate-buffered saline (PBS) and resuspended in PBS at 25% SRBC. Each fish received an intraperitoneal injection of the SRBC suspension (0.1 ml SRBC suspension/ 16 g body mass). After 14 days we collected 20-100 µl blood from the caudal vein with a 1 ml syringe. The blood was mixed with a drop of heparin (<1 µl) and centrifuged at 13,000 rpm for 10 min. The blood plasma was stored at -20°C until the haemagglutination test and quantification of 11-KT levels. Pre-immunization blood samples for the haemagglutination test were not taken, since antibody titer is zero for un-immunized *Pundamilia* cichlids (N=11, Dijkstra unpublished). Antibody concentrations in 20 µl of plasma were estimated in a standard haemagglutination titration assay (Hudson & Hay 1989). To prevent lysis of sheep red blood cells by complement, the plasma was heated to 56°C for 30 min (Collazos et al. 1994). Thereafter, plasma was diluted 1:1 in PBS and then serially diluted in PBS in 96-well microtiter plates. An equal volume of 0.2% SRBC was added to these dilutions, and the plates were incubated at 37°C for 60 min. Titers were scored visually as the highest two-fold dilution of plasma showing haemagglutination. Scoring was done blind with respect to treatment and color score of the males.

**Plasma 11-KT analysis**

Quantification of 11-ketotestosterone plasma levels (the most important androgen in fish, Kime 1993) by radioimmunoassay was carried out as described previously (Schulz et al. 1993), except that smaller plasma volumes were available (20-40 µl). Therefore, the effective lower limit of detection for 11-KT in individual samples was 0.48 ng/ml plasma. The intra-assay coefficient of variation of a pooled plasma sample containing 10 ng/ml was 13%. All plasma samples were analyzed in a single assay.

The individual samples (20-40 µl of plasma) were diluted with a two-fold volume of an aqueous sodium azide solution (0.05%, w/v), incubated at 80°C for 1 hr to liberate protein-bound steroid hormones, and then centrifuged at 14,000 rpm for 30 min at room temperature. The supernatant was harvested and stored at 4°C. The same day, 50 µl of the supernatant was analyzed for its content in 11-KT, using a radioimmunoassay (Schulz 1985). The details of antibody specificity and cross-reactivities are given in Schulz (1985).

**Statistics**

We used a repeated measure ANOVA (RM-ANOVA) to analyze the development of red score over the experimental period, with treatment as fixed factor and day as
repeat. We removed one non-stimulated male from the RM-ANOVA, because he
was not photographed on the first day. We collected blood from 13 males from the
stimulated group, and 13 from the non-stimulated group for the haemagglutination
test. Of these individuals, we collected enough plasma of 9 stimulated and 11 non-
stimulated males for conducting the RIA assay too. For the analysis of antibody
production, two animals, a single individual in both groups, were left out of the
analysis because they did not show any antibody response.

We used a backward stepwise selection procedure to determine if the red
score prior to immunization on day 10 (pre-immunization red score) was a
predictor of the antibody response. The full model also included treatment and the
interaction term of treatment and pre-immunization red score. The criterion to
remove a variable was set at $P=0.15$ to reduce the probability of making type II
errors (incorrectly failing to reject the null hypothesis).

To analyze whether 11-KT was related to antibody production, we carried
out backward and forward selection procedures, using the subdataset that included
males with known 11-KT levels. Again, the response variable was antibody
production. The full model included the red score on day 25 (final red score, which
is closest in time to blood collection and therefore likely to be related to 11-KT
levels), 11-KT level, treatment and all two-way interaction terms. Again, the
criterion to remove a variable was set at $P=0.15$, starting with the interaction terms.

In all models we also analyzed the effect of weight of the test fish. We did
not include weight in the starting model in the interaction terms. Weight never
explained a significant part of the variation in the response variable ($Ps>0.4$). The
same was done with the amount of plasma we collected: the amount of plasma
never explained a significant part of the variation in the response variable ($Ps>0.8$).
All percentage variables were arcsine square root transformed prior to use in
parametric tests. All other variables did not require transformation to meet
assumptions of parametric testing. Quoted probabilities are for two-tailed tests of
significance. We report means and ± standard errors.

Figure 1 Final red score (day 25) in
relation to rank (one is the most
dominant, eight the most
subordinate, $N=32$) for four groups,
each indicated by a different symbol.
Overlapping points have been
slightly displaced for visual clarity.
Asterix indicates four exactly
overlapping data points. The
regression line is fitted for all groups
combined.
Results

Experiment I

On average, males were involved in 11.3 aggressive interactions/5 min (N=32, SE=3.9/5 min). Redder males had higher dominance (Figure 1, Fisher’s Combination Test, $\chi^2=19.55$, df=8, P=0.012).

Experiment II

Nuptial coloration

At the start of the experiment (day 0), red score did not differ between the treatment groups (Figure 2, independent t-test: $t_{28}=0.459$, P=0.65). On day 4 stimulated males increased in red score relative to day 0 (paired t-test: $t_{15}=-6.833$, P<0.001), whereas there was no detectable change in red score in the non-stimulated males (paired t-test: $t_{13}=0.269$, P=0.80). The change in red score over the entire experimental period was significantly higher in the stimulated group than in the non-stimulated group (RM-ANOVA: treatment $F_{1,23}=25.276$, P<0.001; day effect, repeat $F_{1,23}=12.372$, P=0.002; repeat x treatment $F_{1,23}=6.892$, P=0.015). From day 4 onwards stimulated males maintained a substantially higher red score than the non-stimulated males (independent t-tests: all days Ps<0.001). Thus socially-stimulated males rapidly showed elevated sexual signaling relative to non-stimulated males.

Antibody response and nuptial coloration

Antibody titers were on average lower but not significantly so in the stimulated group ($6.5 \pm 0.9$, N=12) than in the non-stimulated group ($7.8 \pm 0.4$, N=12, independent t-test: $t_{22}=1.254$, P=0.22).
Figure 3 Antibody production in relation to pre-immunization red score for stimulated males (closed circles, solid line, N=12), and non-stimulated males (open circles, dashed line, N=12). The final model contained treatment, red score and the interaction term between red score and treatment as significant predictors of antibody response: results of this model are summarized in table 1.

Figure 4 Final red score in relation to 11-ketotestosterone (ng/ml) for stimulated males (closed circles, solid line, N=9), and non-stimulated males (open circles, dashed line, N=12).
We tested if pre-immunization red score was a predictor of antibody production. The backward selection procedure retained all variables in the final model ($F_{3,20}=3.612$, $P=0.03$) with treatment, red score and the interaction between treatment and red score as significant predictors of antibody response (Figure 3, table 1). After removing the interaction term between treatment and red score, neither red score ($F_{1,21}=2.727$, $P=0.11$) nor treatment ($F_{1,21}=0.084$, $P=0.78$) affected antibody response. Overall, red score and antibody response correlated negatively (Figure 3, Pearson's correlation test: $r=-0.515$ $P=0.01$, $N=12$). Within the stimulated group, red score and antibody response correlated negatively (Figure 3: $r=-0.615$ $P=0.033$, $N=12$) but this correlation was absent in the non-stimulated group (Figure 3: $r=-0.077$, $P=0.81$, $N=12$). These findings show that expression of red nuptial coloration correlated negatively with antibody response, but that this negative correlation is dependent on the manipulated social environment.

**Hormones**

Social stimulation led to a higher plasma level of 11-KT (stimulated group: mean ± SE=9.7 ± 2.0, $N=10$, non-stimulated group: mean ± SE=3.4 ± 0.8, $N=12$, independent t-test: $t_{18}=-3.195$, $P<0.01$).

11-KT and red score did not correlate significantly so (Pearson's correlation test: $r=0.416$, $P=0.068$, $N=20$). This was also the case in the stimulated group.

![Figure 5 Antibody production in relation to 11-ketotestosterone (ng/ml) for stimulated males (closed circles, solid line, $N=10$), and non-stimulated males (open circles, dashed line, $N=11$).](image-url)
(r=0.655, P=0.055, N=9), as well as in the non-stimulated group (Figure 4, r=-0.434, P=0.18, N=11).

Table 1 The final ANOVA model after the backward selection procedure, testing whether pre-immunization red score was a predictor of antibody production. For the full model, see text. The final model retained the effect of treatment (stimulated and non-stimulated), pre-immunization red score and the interaction effect on antibody response (see also figure 3).

<table>
<thead>
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<th>Source</th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>1</td>
<td>4.875</td>
<td>0.039</td>
</tr>
<tr>
<td>Red score</td>
<td>1</td>
<td>5.280</td>
<td>0.032</td>
</tr>
<tr>
<td>Treatment x red score</td>
<td>1</td>
<td>5.471</td>
<td>0.030</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

To test if 11-KT is related to antibody production (Figure 5), we carried out a stepwise selection procedure on a subset of the data, i.e. those cases with 11-KT information. The full model, with response variable antibody production, included final red score, 11-KT level, treatment and the two-way interaction terms. The procedure yielded a nonsignificant model (effect of 11-KT F₁,₁₈=1.531, P=0.232). Since 11-KT levels showed a not significant trend to correlate with final red score, we regressed 11-KT levels against final red score for each treatment separately. These residuals of 11-KT were used in the starting model instead of 11-KT level. This analysis confirmed that 11-KT was not correlated with antibody response (residuals of 11-KT F₁,₁₇=0.391, P=0.54). In addition, forward selection procedures, both using either the residuals or 11-KT levels, confirmed that 11-KT did not predict antibody production (Ps>0.23).

Discussion

**Male-male competition stimulates sexual advertisement**

Socially dominant males expressed enhanced red nuptial coloration in a male-male competition context (experiment I). Bright red coloration may therefore be important in intrasexual signaling, indicating social dominance, competitive ability or territory ownership (Rohwer 1975; Maynard-Smith & Harper 1988). Social stimulation with a rival male resulted also in significantly larger areas of red coloration (experiment II). This effect was sustained for the duration of the experiment. The finding concurs with other studies showing that animals modify color expression with respect to the social environment (e.g. sticklebacks: Candolin 1999; zebra finch: McGraw & Ardia, 2003). Lighting conditions differed between our study and the study of Maan et al. (2004), precluding a direct comparison between field and laboratory data. A quantitative assessment of color scores on a 0-
5 scale indicates that color scores of the stimulated and the non-stimulated males correspond to those found in the field for territorial and non-territorial males respectively (M. E. Maan, personal communication). Territorial *P. nyererei* males typically express bright red coloration, whereas non-territorial males appear more cryptic.

Maan et al. (2004) showed that *P. nyererei* females preferred bright red males. The present study underscores the dual role of red coloration as a signal in both mate choice and male-male competition (Berglund et al. 1996), supporting the idea that male-male competition affects mate choice (Wong & Candolin 2005).

**Trade-off between sexual signaling and immunocompetence**

A central prediction of the immunocompetence handicap hypothesis is that a trade-off exists between sexual ornamentation and immune function. We found an overall negative correlation between red score and humoral response, in agreement with the prediction. However, the experiment failed to reveal an overall treatment effect on antibody production, whereas it did affect color display. Nonetheless, the manipulation interacted significantly with red score: only in the stimulated group did red score correlate negatively with antibody production. This lack of a negative correlation in the non-stimulated group was probably attributable to the fact that males in this group did not reach relatively high red scores. Thus we showed that the negative correlation between coloration and antibody production is dependent on the manipulated social environment, lending experimental support to the hypothesis that color expression goes at the expense of a humoral immune response to a foreign antigen. The research on this trade-off has been skewed towards birds (Roberts et al. 2004). This study provides some of the first experimental evidence for a trade-off between expression of a sexual trait and one component of immune function in fish (see also Grether et al. 2003). It is possible that this mechanism reinforces honest status-signaling in *P. nyererei*, important in both mate choice and rival assessment.

We probed only one component of the immune system, namely the humoral response to a foreign antigen. Antibody responsiveness to SRBC is known to correspond with resistance to several important parasites in some vertebrates (e.g. Parmentier 1996). Yet, future studies on the trade-off between sexual display and immune function should examine other immune variables, such as antioxidant activity, phagocytosis and response to naturally occurring parasites (Adamo 2004). Similar data have been obtained in the Azorean rock-pool blenny (*Parablennius parvicornis*) where males adopt either a parasitic or a bourgeois mating tactic (Ros et al. 2006). Parasitic males are nonterritorial and have low levels of 11-KT, whereas Bourgeois males are territorial and have elevated levels of 11-KT. The bourgeois mating tactic is associated with more intense sexual ornamentation, and in agreement with the prediction, antibody response to (SRBC) was lower in parasitic males than in bourgeois males (Ros et al. 2006).
Interestingly, in a field study on *P. nyererei*, intensity of male red coloration correlated negatively with parasite load (Maan et al. 2006c). Their study shows that bright red males are less parasitized, which is unexpected in view of the immunological cost to expressing red nuptial coloration. A similar contrast has been found previously in a bird species (Peters 2000). The contrast can adequately be explained by assuming that in unmanipulated animals only good quality individuals are able to advertise full coloration, while in the experimental study colour enhancement was imposed upon males.

The haplochromine cichlids represent one of the best test cases of sympatric speciation by sexual selection on male trait through divergent female mate choice (Seehausen 2000; Kocher 2004). Theory suggests that ‘good genes’ processes are less likely to drive sympatric speciation than divergent Fisherian runaway selection (Van Doorn et al. 2004; Kirkpatrick & Nuïmer 2004). This is because Fisher runaway process may unfold in any arbitrary direction, whereas in ‘good-genes’ models of sexual selection, female mate choice is less arbitrary and should be directed to traits that reliably signal genetic quality of the male. Our study adds experimental support to the hypothesis that haplochromine males advertise ‘good genes’, representing another possible example of numerous studies describing sexually selected traits that reliably indicate genetic quality (for reviews see Andersson 1994; Candolin 2003; Neff & Pitcher 2005). It thus appears that purely Fisherian traits are an unrealistic assumption in many speciation models (Turelli et al. 2001). Since the trait under study, male nuptial coloration, has been implicated in the rapid haplochromine species radiation (Seehausen 2000; Kocher 2004), it is necessary to improve our understanding of the mechanisms that may lead to sympatric divergence in the face of ‘good-genes’ sexual selection (see also Edelaar et al. 2004).

**Androgens**

Social stimulation led to higher plasma levels of 11-KT. This is consistent with the observation that in a number of cichlid genera, male-male interaction leads to a physiological maximum of 11-KT. (Hirschenhauser et al. 2004). The levels of 11-KT of the stimulated males and the non-stimulated males correspond to those documented in *Haplochromis burtoni* of dominant and subordinate males respectively (Parikh 2006). Sexual advertisement is stimulated by androgens (Andersson 1994), as has also been demonstrated in cichlids (Fernald 1976, Groothuis & Ros unpublished) and even in *Pundamilia* (Pardijs, Dijkstra & Groothuis unpublised). The results are consistent with this notion. Firstly, social stimulation led to a parallel increase in androgens and sexual signaling. Secondly, the relationship between androgens and red score was positive, although this was marginally nonsignificant.

The evidence for a causal link between elevated androgens levels and immuno-suppression is ambiguous in fish. In salmonids, several studies suggest that there is a direct pathway for androgen induced immuno-suppression (Slater &
Schreck 1993; Slater et al. 1995a,b; Slater & Schreck 1998). Studies in other teleosts have been unsupportive (Uglem et al. 2001; Law 2001). Evidence is accumulating that the immuno-modulating effects of androgens are mediated by indirect effects of androgens via regulation of resource allocation of for example carotenoids (Wedekind & Folstad 1994; Blount et al. 2003), or via its effect on glucocorticoid hormones (e.g. Hillgarth & Wingfield 1997). Law et al. (2001) found a suppressive effect of cortisol on leukocyte phagocytosis, but androgens had no effect. Experimental elevation of androgen levels is necessary to address this question further. The interaction effect of treatment and color expression on antibody production, with the former affecting both color expression and 11-KT levels, indicates that androgens, color expression and antibody production are interdependent.

Conclusions

Our experiment illustrates the importance of male-male competition in sexual signaling, suggesting that red nuptial coloration is a signal to both prospective partners and rivals. We found both correlational and experimental evidence for the hypothesis that males face a trade-off between color expression and one component of immunological defense. Carotenoid-dependent pigmentation in *P. nyererei* is therefore likely to be a costly trait, enforcing honest advertising of a male’s quality. This has implications for the involvement of coloration and sexual selection in speciation.

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Female-female aggression can promote stable color polymorphism

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Abstract
The co-existence of ecologically similar species has been a longstanding problem in evolutionary ecology. Theory and experimental work suggest that co-existence is promoted if territorial males bias aggression towards phenotypically similar rivals, generating negative frequency-dependent selection between species. We propose that where females are territorial, the same mechanism in females may promote species co-existence too. We studied a haplochromine cichlid species from Lake Victoria, *Neochromis omnicaeruleus*, in which three distinct female color morphs co-exist. In the laboratory we tested for aggression biases in wild-caught females of each morph with stimulus females of every morph, enclosed in transparent tubes. Females of each morph biased aggression towards stimulus females of their own morph, suggesting that females of all three morphs may have an advantage when their morph is locally the least abundant. This mechanism may contribute to stabilizing the color polymorphism. The absence of aggression bias in blotched and plain sisters bred by crossing a blotched and a plain parent suggest that genetic linkage cannot explain the observed association between color and aggression bias in nature despite hybridization between morphs. We discuss alternative hypotheses that could explain the association. We conclude that female-female aggression may be an important force for stabilizing speciation and species co-existence.
Introduction

The colorful haplochromine cichlid species flocks of the Great African Lakes are the most extreme examples of explosive speciation among vertebrates. Most of the astounding species diversity in Lake Victoria likely evolved from a few ancestors in the past 15,000 years (Johnson et al. 1996; 2000; Seehausen 2006a). A great diversity of trophic adaptations has evolved rapidly, associated with strong habitat and trophic resource partitioning among the major lineages (Seehausen 1996; Seehausen et al. 1999a). However, many immediate sister species co-exist in sympathy with apparently little, if any ecological differentiation but with marked differences in male nuptial coloration (Bouton et al. 1997; Seehausen et al. 1999a,b). The same pattern repeats itself in the older radiation of haplochromines in Lake Malawi (Seehausen et al. 1999a; Danley & Kocher 2001; Kocher 2004; Genner & Turner 2005). The sympatric co-existence of ecologically similar species that differ mainly just in nuptial coloration (or color morphs in the case of ongoing gene-flow) is a longstanding problem in ecology (in haplochromines: e.g. Reinthal 1990; Seehausen 1996; Bouton et al. 1997; Genner et al 1999a; 1999b; Genner & Turner 2005; other: e.g. Hutchinson 1961; Scheffer & Van Nes 2006). One idea is that negative frequency-dependent selection on male nuptial coloration may arise from male-male aggression, thereby promoting species co-existence (Mikami et al. 2004; Seehausen & Schluter 2004; this thesis). Haplochromine males vigorously defend long-term mating territories to secure spawnings (Parker & Kornfield 1996; Maan et al. 2004). Competition over territories is intense, and is likely to influence male fitness. Negative frequency-dependent selection on color could arise if territorial males direct more aggression to phenotypically similar than to dissimilar rivals. Such an aggression bias would lead to rare phenotypes sustaining less intense competition, and thereby enjoying a fitness advantage relative to the more abundant phenotype. Experimental evidence confirms that males of some haplochromine cichlids species preferentially direct aggression towards males of their own species (chapter 2 & 3). Comparative evidence suggests that such interaction may structure haplochromine species composition in nature (Seehausen & Schluter 2004).

In haplochromine cichlids female-female competition is likely to affect female fitness as well. Haplochromines are female mouth brooders and maternal fry guarders. Females aggressively fend off small territories to protect their fry. In algae-scraping species, such as Neochromis omnicaeruleus, females often occupy short term territories over rocky sites to secure access to algae (Seehausen 1996). Finally, territories can also provide shelter against piscine and avian predators. Therefore, interference competition among females for territories is likely, and could exert selection on female coloration too. Females of most haplochromine cichlid species are cryptically colored, consistent with their larger role in parental care (Fryer & Iles 1972; Seehausen 1996). Due to this crypsis, females of closely related species often look phenotypically similar, providing less opportunity for
color-based recognition of competitors and aggression biasing. However, conspicuous female color phenotypes are common in several haplochromine species (Lande et al. 2001), including the highly polymorphic species *Neochromis omnicaeruleus* (Seehausen et al. 1999b). In its Makobe island population, three distinct color morphs occur in both sexes: a plain presumably ancestral morph (P), and two conspicuously colored blotched forms, white blotched (WB; black blotches on white) and orange blotched (OB; black blotches on orange). The WB and OB morph is predominantly found in females. Intermediates between these morphs exist but are rare. A long term series of field data from 1991 to 2003 suggests morph frequencies have fluctuated little over at least 12 years (Seehausen et al. 1999b; Maan 2006). Females are quite aggressive and territorial too (Seehausen et al. 1999b; Maan 2006). The existence of intermediate phenotypes suggests there is gene-flow between morphs (Seehausen et al. 1999b), and their microhabitat distribution is completely overlapping (Seehausen & Bouton 1997), raising the question how this color polymorphism is maintained (Seehausen et al. 1999b; Lande et al. 2001).

We tested in wild-caught females of *Neochromis omnicaeruleus* whether territory defenders bias aggression towards competitors of their own color morph, using a simulated intruder choice test (chapter 2, 3 & 5). We show that females of each color morph differ in aggression bias in the predicted direction: females of each morph exhibit an own-morph bias in aggression, which may generate negative frequency-dependence on female coloration. This then raises the question how the association between color and aggression bias is maintained in the face of gene-flow. Gene-flow would quickly erode any associations between color and aggression bias genes by assortment and recombination. We tested whether genetic linkage (via pleiotropy or physical linkage) between color and aggression bias could maintain the own-morph bias in aggression. We examined aggression biases of plain and blotched sisters bred from a plain and a blotched parent. Genetic linkage, but not independent inheritance, predicts own-morph aggression biases in both plain and blotched sisters to be retained, and hence a difference in aggression bias between the two morphs.

**Methods**

**Species**

We studied a population of *Neochromis omnicaeruleus* from Makobe Island in the western Speke Gulf (Tanzania) that consists of three fully sympatric morphs. Biogeographical data, field observations and laboratory experiments suggest that plain (P) is the ancestral morph, and orange blotched (OB) and white blotched (WB) are two derived morphs or incipient species at Makobe Island (Seehausen et al. 1999b). P females are yellow to brown with 4–8 dark vertical bars, whereas most P males are blue and some are yellow. OB individuals have variably shaped brown
blotches, superimposed on an orange or pink background. WB individuals have
variably shaped black blotches on a white to brassy background. Even though half
of the females in the population are blotched, only less than 1.7% of the males are
blotched. The three morphs are fully sympatric and ecologically indistinguishable,
but partially reproductively isolated by male and female mating preferences. Males
(and not females) of the blotched morph exhibit mating preferences against the P
morph, and males and females of the P morph exhibit strong mating preferences
against the blotched morphs (Seehausen et al. 1999b). Nevertheless, intermediate
phenotypes occur, suggesting gene flow.

Subjects and housing
Females were collected in Lake Victoria around Makobe Island, and transported to
the laboratory in Haren, The Netherlands, in February 2003. We tested 15 OB, 16 P
and 15 WB morph females, all wild-caught. We generated plain and blotched full
sib sisters by crossing a blotched with a P parent (blotch is X-linked; Seehausen et
al. 1999b). The laboratory crossings are summarized in table 1. With one exception,
we used different wild-caught fish for each cross (table 1). We made two cross types:
OB female with P male (OBxP), and WB female with P male (WBxP). We also made
one cross between a P female and a WB male. All five OBxP families contained P
(henceforth Pob) and OB females, and P males (table 1). All five WBxP families
contained P (henceforth Pwb) and WB females, and P males, consistent with an
existing model of inheritance (Seehausen et al. 1999b). In one of the WBxP families
and in the PxBW family we also had one WB male each (table 1).
Females grew up in sib-groups, guarded by their mother for the first four
weeks post-hatching. We removed males as soon as the sexes differentiated around
6 months of age. When individuals began to mature at an age of 4-5 months, we
added 10-15 juvenile *Pundamilia* sp. to each family to disperse aggression. *Pundamilia*
are less aggressive than *Neochromis omnicaeruleus*, and are
commonly used as dither fish in our breeding aquaria. We tested 18 OB, 13 Pob, 22
WB and 16 Pwb lab-bred females. The number of females tested per sib-group is
shown in table 1.
All aquarium walls, except the front, were covered with black plastic sheets.
All aquaria were connected to a central biological filtration system and water
circulated continuously. Water temperature was kept at 25 ± 2°C and a 12:12 h
light:dark cycle was maintained. The bottom of the aquaria was covered with gravel.
Fish were fed flake food (Tetra Min Tropical Fish Flakes) seven times per week.

Pre-experimentation housing
For at least one week prior to the experiments females were individually housed in
separate compartments with a PVC tube as a refuge. Approximately ten
compartments were made in 100-170 liter aquaria using transparent perspex sheets.
Females had one or two direct neighbors, and visual access to all other females in
the tank. The arrangement ensured that females became territorial and avoided unwanted effects of social isolation, while preventing them to engage in physical interaction. Wild-caught females were visually exposed to females of all three morphs. Lab-bred females were visually exposed only to females of their own morph and that of their sisters. Test females were never housed adjacent to the corresponding stimulus females.

Table 1: Summary of the crosses to generate lab-bred plain and blotched females. Shown are the family code, morph of mother, father, the number of sons and daughters. Note that these numbers represent the moment when females were transferred from sib-groups to the pre-experimentation housing. Numbers at the moment of fry release were different due to mortality. Also shown are the number of plain and blotched daughters that we tested. All parents were wild-caught, unless indicated otherwise (F1=mother bred from wild-caught parents).

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1) This female bred twice with two different males: broods were raised together in a single aquarium
2) This is a combined brood of two females and a single male: broods were raised together in a single aquarium
**Simulated intruder choice test**

We used a simulated intruder choice test to estimate aggression biases. Test females were allowed to become territorial, and were then presented with stimulus females in paired combinations (WB-OB, P-OB and P-WB). Wild-caught test females were tested only with stimulus pair combinations that contained their own morph, thus each wild-caught female was presented with two stimulus pairs. These two stimulus pairs were selected from standard length matched stimulus trios (OB, P and WB). Lab-bred P_ob and OB were tested with P-OB stimulus pairs, and lab-bred Pwb and WB were tested with P-WB stimulus pairs. No stimulus pair was used more than once with either morph. A test aquarium consisted of a large experimental compartment (55 x 35 x 39 cm, l x w x h) for the test fish, and a smaller compartment (5 x 35 x 39 cm) for a dither neighbor, needed to maintain territorial condition of the test fish. The dither neighbor was a small female of an unrelated Lake Victoria cichlid (*Pundamilia nyererei* [Witte-Maas & Witte, 1985]). It was separated from the test fish by a transparent partition. Test females were transferred from the pre-experimentation housing aquarium to the experimental compartment one day before a trial to allow acclimatization to the test aquarium. A PVC tube was provided as a refuge.

At the start of a trial, two stimulus females, individually confined in transparent watertight Perspex tubes, were placed in the experimental compartment 20 cm apart from one another and at the end of the tank opposite to the neighbor fish. No chemical communication was possible between stimulus and test fish. The test females perceived the stimulus females as intruders, and typically responded aggressively to both stimulus females, whilst the neighboring *P. nyererei* female was entirely neglected. We recorded the number of attacks of the test female to each of the stimulus females for five minutes starting directly after introduction of the stimulus females. An attack was defined as biting or butting at the walls of the tube containing the stimulus female. An attack was terminated with a display event, or when the test female turned away from the stimulus female (chapter 3). Across trials we randomly assigned stimulus females to left and right positions. The sequence in which the two stimulus pair combinations were presented to wild-caught test females was randomized too. After a female had been tested, she was used as a stimulus female to test other females. The interval between testing a female, and using her as a stimulus was at least one day. Every female was tested before she was used as a stimulus.

**Analysis**

A female’s aggression level was estimated by the total number of attacks she launched in 5 minutes averaged over all trials of that female. We tested for differences in the level of aggression between morphs using ANOVAs.

Aggression biases could be expressed in two ways. The attack ratio towards females of a particular morph was calculated as the number of attacks
launched against the stimulus females of that morph divided by the total number of attacks launched to both stimulus females in the same trial. A similar aggression bias was calculated as the *attack ratio towards females of the own morph*. We first tested whether females differed in attack ratio using independent t-tests. We then tested whether females had a significant aggression bias towards their own color morph, using a doubly nested repeated-measure ANOVA (RM-ANOVA), with ‘morph of test female’ (3 levels) as explanatory variable. The first repeat (referred to as ‘stimulus pair’) consisted of attack ratios towards own morph for the two stimulus female pairs with which a test female was tested. The second repeat (referred to as ‘bias’) was nested within the ‘stimulus pair’ repeat. It evaluated aggression biases by testing whether the attack ratio towards own morph deviated significantly from 50%. We also computed an RM-ANOVA for females of each morph separately. The results of the ‘stimulus pair’ repeat are not involved in any hypothesis testing, and is therefore not reported. Separate post-hoc tests to evaluate aggression biases for each morph and stimulus pair combination separately was done by testing the attack ratio against 50% using paired t-tests.

For the analysis of the lab-bred females, we first tested for each cross type (WBxP or OBxP) separately whether family explained any significant fraction of the variance in the attack ratio. To this end, we used linear hierarchical models (Bryk & Raudenbush 1993) in MLWin 2.0, using a two level model with individual fish nested within families. The response variable was the attack ratio towards P morph, and the explanatory variable was ‘morph of test female’. Family explain none of the variance in attack ratio in any of the cross types (family in both cross types: Ps>0.5). We therefore treated each female as an independent data point in subsequent data analysis. We tested for genetic linkage between color and aggression bias against the alternative of independent inheritance of both traits by comparing the attack ratio of plain and blotched females towards P using independent t-tests. A difference in aggression bias would support genetic linkage.

One wild-caught WB female and one lab-bred WB and OB female launched no attacks at all and were not included in the analysis of aggression bias. To meet assumptions of parametric testing, we arcsine square root transformed the attack ratio data, and square root transformed the aggression level data. All reported probabilities are for two-tailed tests. Statistical analyses were performed in SPSS 12.0.1 unless stated otherwise.
Figure 1A The attack ratio (mean ± SE) of wild-caught females of each color morph (indicated at the bottom) for each stimulus pair combination (indicated on top). The attack ratio is expressed as the proportion of attacks towards one morph (P in the P-OB and P-WB combination, and WB in the WB-OB combination) relative to the total number of attacks. An attack ratio of 0.5 represents identical numbers of attacks to both stimuli (-----). Indicated are differences in attack ratio between females of different morphs and significant deviations from 50% (for statistics see text). (*P<0.05, **P<0.01, ***P<0.001).

Figure 1B Same as 1A, but here the attack ratio is expressed as the proportion attacks to one morph.
**Aggression bias in wild-caught females**

The aggression level of OB females was 43.9 ± 5.7 attacks/5 min (mean ± standard errors), that of P females was 47.3 ± 5.7, and that of WB females 38.7 ± 4.7. These differences were not statistically significant (ANOVA F_{2,43}=0.65, P=0.53).

The mean aggression biases towards a particular morph of wild-caught females are shown in figure 1A. Females of different color morphs differed in aggression biases. This was revealed by testing within each stimulus pair combination the bias of females of both morphs as expressed as attack ratio towards females of the same morph (Figure 1B, table 2). Thus, P and OB differed significantly in aggression bias when tested with a P-OB stimulus pair (independent t-test: t_{29}=-2.84, P<0.01), P and WB females too differed when tested with a P-WB stimulus pair (t_{30}=-4.23, P<0.001), and finally, WB and OB females differed when tested with a WB-OB stimulus pair (t_{29}=2.28, P<0.05). All differences were in the direction predicted by own-morph aggression bias.

The mean aggression biases towards own morph of wild-caught females are presented in figure 1B for each stimulus pair combination. Overall, females preferentially attacked their own morph ('bias' repeat [against 50%], F_{1,42}=23.24, P<0.001). The magnitude of this own-morph bias was not different between morphs (Figure 1, morph of test female, F_{2,42}=0.68, P=0.51).

Repeating the same nested RM-ANOVA for females of each morph separately detected significant own-morph biases in aggression in P and OB females, but not in WB females (OB: F_{1,14}=7.4, P=0.02, P: F_{1,15}=64.1, P=<0.001, WB: F_{1,13}=2.1, P=0.17). Further post-hoc tests consisted of testing for each morph and stimulus pair combination separately if the attack ratio deviated from 50% (table 2). Significant own-morph biases were retained for OB females when presented with an OB-P stimulus pair, and for P females when presented with a P-WB stimulus pair (see figure 1B).

**Aggression bias in lab-bred females: genetic linkage?**

The aggression level of P_{ob} females was 32.6 ± 6.3 attacks/5 min (mean ± standard errors), that of OB females 33.2 ± 6.1, of P_{wb} females 34.6 ± 6.0 and of WB females 27.3 ± 4.3. There was no difference in aggression levels between females from OBxP families and females from WBxP families (ANOVA, cross type [OBxP versus WBxP]: F_{1,65}=0.084, P=0.77), nor between blotched and plain individuals (ANOVA, color morph [blotched versus plain]: F_{1,65}=0.639, P=0.43). Neither did the interaction term between both factors contribute significantly to the explained variance (cross type x color morph: F_{1,65}=0.341, P=0.56).

The mean attack ratio towards P is shown in figure 2A, and towards own morph in figure 2B. Genetic linkage between color and aggression bias predicts a difference in aggression bias between blotched and plain females. Neither P_{ob} and OB (independent t-test: t_{28}=1.38, P=0.18), nor P_{wb} and WB (t_{35}=-0.293, P=0.77)
differed in the attack ratio towards P (Figure 2A, table 2). The results make genetic linkage unlikely explanations for the own-morph aggression biases observed in wild-caught females.

![Graph A](image1.png)

**Figure 2A** The attack ratio (mean ± SE) for lab-bred OB and P_ob females derived from OBxP families (left panel), and for lab-bred WB and P_wb females derived from WBxP families (right panel). The attack ratio is expressed as the proportion of attacks to P relative to the total number of attacks. An attack ratio of 0.5 represents identical number of attacks to both stimuli (-----). Deviation from 50% is tested using paired t-tests. None of these was significant. There was no differences in attack ratio between morphs (NS=nonsignificant, for statistics see text).

![Graph B](image2.png)

**Figure 2B** Same as 2A, but here the attack ratio is expressed as the proportion attacks to own morph.
We tested for females of each morph separately whether lab-bred females preferentially attacked females of their own morph by testing whether the attack ratio deviated from 50% (Figure 2A, table 2). None of the female groups showed any preference to attack own morph, whereas WB females showed a nonsignificant tendency to preferentially attack P females.

Table 2 The aggression bias expressed as the attack ratio towards one morph for wild-caught and lab-bred females, calculated as the number of attacks to one morph divided by the total number of attacks (equivalent to figure 1B and 2B, see also note). Indicated are means ± SE. To assess aggression bias, the attack ratio is tested against 50% using paired t-tests. Shown are the t statistic and the P value. Significant effects are indicated in bold.

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Discussion

Using a simulated intruder choice test and wild-caught fish, we found aggression biases towards own morph among female color morphs of *Neochromis omnicaeruleus*. These results suggest that in competition for territories, females of all three morphs may receive fewer attacks, and thereby experience elevated fitness when their morph is locally the least abundant of the three. Elevated fitness may
come about via a higher probability to gain access to limiting food resources, or shelter from predators. Shelter is important both for females themselves and for their fry. The negative frequency-dependent selection that is expected to arise from this own-morph aggression bias may both facilitate the invasion of a novel color type, and stabilize a color polymorphism once it is there (Mikami et al. 2004; Seehausen & Schluter 2004).

Why do females of *Neochromis omnicaeruleus* bias aggression towards self-colored females, even though all three morphs compete for the same ecological resources (Seehausen & Bouton 1997; Seehausen et al. 1999b)? We can only speculate about this question. Perhaps the answer lies in mutual mate choice and female competition for mates. In *Neochromis omnicaeruleus*, males (and not females) of the blotched morph exhibit mating preferences against the P morph. Females and most males of the P morph exhibit strong mating preferences against the blotched morphs (Seehausen et al. 1999b; Pierotti & Seehausen 2006). Mutual mate choice would render females of the same morph competing more strongly for males with the same preference (see also Lande et al. 2001). Therefore, females would benefit from directing more aggression to rival females of their own morph with whom they share the same potential partners. The choosiness of males suggests that such mate competition among females exists.

The *Neochromis omnicaeruleus* system has inspired models of sympatric speciation by sexual selection (Lande et al. 2001; Kocher 2004, see also Seehausen et al. 1999b; Pierotti & Seehausen 2006). The selective mating among colour morphs led Seehausen et al (1999b) to suggest that the *Neochromis omnicaeruleus* system has properties of an incipient stage of sympatric speciation by sexual selection. Forces that can account for polymorphism in female mating preferences are essential to the process of sympatric speciation by sexual selection (Van Doorn et al. 2004). In this context it is interesting to note that recent theory indicated the importance of female-female competition in generating and maintaining such a polymorphism (Van Doorn et al. 2004). Own-morph aggression biases among females could contribute to the evolution of a female-preference polymorphism by creating negative frequency-dependent selection on female preference.

Given the evidence for gene-flow among the *Neochromis omnicaeruleus* morphs (Seehausen et al. 1999b) the question how the association between color and aggression bias is maintained is intriguing. In theory, if own-morph biases in aggression are expressed pleiotropically with color, the emergence and co-existence of incipient species would be greatly facilitated. Such linkage or pleiotropy between color and preference genes, even though perhaps unlikely at first sight, has recently been demonstrated in *Heliconius* butterflies (Kronforst et al. 2006). We tested for genetic linkage by examining aggression biases of lab-bred plain and blotched sisters from morph crosses, but neither found any difference in aggression bias between plain and blotched females, nor any own-morph bias. Thus, genetic linkage (through pleiotropy or physical linkage) is unlikely to explain the observed own-morph biases in aggression of wild-caught females. The results make the
alternative, independent inheritance of colour and aggression bias more likely. The absence of evidence of genetic linkage suggest that the association between color and aggression bias observed in wild-caught females has to be maintained either by a high degree of assortative mating among morphs, or by strong selection against recombinant phenotypes or both. This is consistent with the hypothesis of Seehausen and co-workers (1999b) that morphs are partially isolated by male and female mating preferences. However, the incompleteness of behavioral isolation and the breakdown of the association between color and aggression bias in the first hybrid generation, leaves questions open as to the level of gene-flow between morphs and the strength of selection against color-behavior recombinants.

In conclusion, our study shows that wild-caught females of *Neochromis omnicaeruleus* bias aggression towards females of their own color morph. Such an aggression bias was originally proposed for competition among males resulting in negative frequency-dependent selection on male colour (Mikami et al. 2004; Seehausen & Schluter 2004; Van Doorn et al. 2004; chapter 2-7). The reproductive output of haplochromine populations is likely limited by survival and energy intake of females, and not much by that of males (Genner et al. 1999b). Territorial interactions between females are thus likely to be important for recruitment of competing color morphs or species, affecting their potential to co-exist. Blotch color polymorphisms are common in cichlids in several African Lakes (Lande et al. 2001; Kocher 2004). It would be interesting to see how common female aggression biases are in these systems.

**Acknowledgements**

We thank Mhoja Kayeba, Mohamed Haluna, Machteld Verzijden, Inke van der Sluijs, Marcel Haesler, John Mrosso and Martine Maan for help with the collection of live fish. Sander van Dijk, Iris Bakker and Anneke Procee are greatly acknowledged for help with experiments. Serge Daan, Albert Ros and Nicolaus Baron von Engelhardt gave useful comments on the manuscript. The research was financed by a NWO (SLW) grant 810.64.013. The research was carried out with an animal experiment license (DEC 2812) from Groningen University and complied with current laws in The Netherlands.
10

General Discussion

Peter D. Dijkstra
In this final chapter I will summarize and discuss the main findings and integrate them in a more general context. I will also give some directions for future research.

**Speciation and intrasexual selection**

The origin and maintenance of biodiversity form a central question in biology (Ricklefs & Schluter 1993). My thesis is concerned with one particular mechanism involved, viz. sympatric speciation driven by disruptive sexual selection by female mate choice on male trait. The role of aggressive male-male competition in exerting frequency-dependent disruptive selection is a crucial feature of this topic (Mikami et al. 2004; Seehausen & Schluter 2004; Van Doorn et al. 2004). I tested this role in one of the best test cases for rapid sympatric speciation by sexual selection, the haplochromine cichlid fish from Lake Victoria (Seehausen 1996; Seehausen & Van Alphen 1999; Maan 2006). In this chapter, I integrate the findings of this thesis in a more general framework.

I distinguish three mechanisms that can generate or contribute to frequency-dependent intrasexual selection leading to an advantage of rare male phenotypes: (1) own-type bias in aggression, (2) dominance through color effects, and (3) dominance through higher intrinsic aggression level. These mechanisms were investigated in males of different *Pundamilia* species representing a common color dichotomy (red/yellow-blue) in haplochromines (Seehausen 1996; Seehausen et al. 1999a). Frequency-dependent intrasexual selection on female nuptial coloration is also possible: aggression bias in aggression was tested in females of *Neochromis omnicaeruleus*, representing a common female color polymorphism in haplochromines (Seehausen 1996; Konings 2001; Lande et al. 2001).

*Same traits important in mate choice and intrasexual signaling?*

I first need to discuss an important assumption tested in males of *Pundamilia*. The idea that rare male advantages can facilitate speciation is based on the assumption that the same traits involved in mate choice are also subject to intrasexual selection (Van Doorn et al. 2004). A combination of selective forces (female choice and male-male competition) acting on the same trait is required to allow for sympatric divergence.

Seehausen & Van Alphen (1998) have shown in *Pundamilia* that females prefer conspecific males under normal light conditions when given a choice between *P. Pundamilia* (blue) and *P. nyererei* (red), but not under monochromatic light which effectively eliminates the color difference between red and blue. This suggests that interspecific mate choice is based male coloration. I used a similar light manipulation in chapters 2 and 4. Chapter 2 reports an experiment in which we measured the attack preference using a simulated intruder choice test in red and blue *Pundamilia* fish. Territory defenders were allowed to attack two size-matched red and blue stimulus males enclosed in watertight transparent tubes.
mimicking intruders. They only discriminated when color differences between red and blue stimulus males were visible, but not when masked by green light. This indicates the importance of nuptial coloration in male-male conflicts. This is also indicated by the finding that red males only dominated blue males in dyadic combat under normal light, but less so under green light (chapter 4). Bright red coloration in *P. nyererei* was correlated with social dominance in groups of males. Also, red coloration increased upon social stimulation by a rival male (chapter 8). Therefore, nuptial coloration is clearly important in both inter- and intrasexual selection, supporting a basic premise of the rare male hypothesis.

**Aggression biases, dominance and aggression levels in males of *Pundamilia***

*Aggression biases varies between stages of speciation*

Integration of the results in chapter 2 and 3 yields a more complete picture of the strength and direction of aggression biases, in different populations of *Pundamilia* (figure 1). I studied three types of populations. (1) a predominantly blue population (single-species population), (2) populations where red and blue are distinct phenotypes, with some hybrids occurring in lower frequency (hybridizing-species populations), and (3) populations where red and blue species are reproductively isolated sympatric species (two-species populations). In the hybridizing-species populations hybridization between red and blue occurs as a result of low water transparency interfering with interspecific mate choice (Seehausen et al. 1997). We took the different types of populations as different speciation stages, with the single-species population representing a pre-invasion stage, the hybridizing-species populations representing an incipient stage of speciation after successful invasion of red, and the two-species populations representing completed speciation. I found own-type biases in males from the single-species population (chapter 3). In two hybridizing-species populations both incipient species biased their aggression towards red (chapter 2 and 3). In three two-species populations both species biased aggression towards conspecifics (chapter 2 and 3). It thus appears that red males always bias aggression towards their own color, often more so than blue males (chapter 2), whereas the aggression bias of blue males depends on the stage of speciation. In conclusion, own-type biases in aggression may facilitate the invasion of novel color types in a predominantly blue population, and stabilize co-existence of reproductively isolated daughter species. This is because own-type biases is expected to lead to a rare male type sustaining less competition than the more abundant males type, thereby generating negative frequency-dependence. Aggression bias alone, however, cannot stabilize an incipient stage of speciation. This lack of own-type bias in blue males from hybridizing-species populations can be explained by considering the degree of competition for females (see chapter 2).
Dominance relationship and stable species co-existence

I took one hybridizing-species population, *Pundamilia* males from Kissenda Island, to test dominance relationships between red and blue males. Red males had a higher chance to win than blue males in a dyadic combat between red and blue, but their advantage was significantly reduced when staging combats under green light. This suggests the intimidating nature of red coloration (chapter 4). A similar intimidating effect of red coloration on social dominance has been documented in other animal species (Barlow 1983; Evan & Norris 1996; Pryke & Andersson 2003) as well as for human combat sports (Hill & Barton 2005).

The dominance of red is consistent with the geographic distribution pattern of red and blue *Pundamilia* types. Red types always co-occur with blue, whereas purely blue populations are not uncommon (Seehausen & Van Alphen...
indicating repeated invasion of red phenotypes in blue populations. A dominance advantage of red may have facilitated their establishment. Red males may be subsequently kept at bay since red males incur more attacks than blue males from both red and blue males (chapter 2 & 3). This overall aggression bias towards red males may reflect a secondary adaptation of blue males against dominant red males. The combined effect, i.e. overall aggression bias towards red counteracted by behavioral dominance of red over blue males, may contribute to stable co-existence of incipient red and blue species.

Entirely red populations are not known in *Pundamilia*. This suggests that red males can never displace blue males even though red males dominate blue ones in dyadic combat (chapter 4). Indeed, in groups of red and blue males, both species did equally well in terms of social dominance (chapter 6). In the same experiment the aggression level was higher in groups of only red males than in groups of only blue males. This is consistent with the higher level of aggression in red than in blue reported in chapter 2. I hypothesize that the contrast in dominance of red over blue between a group and a dyadic context is due to the fact that in a group situation red males not only experience aggressive encounters with blue males, but also with other red males, with the latter conflicts possibly being more intense and entailing higher costs. The disproportionate aggression costs that red males incur in a group may weaken the dominance advantage of red over blue males in dyadic combat. The reduced dominance of red over blue in a group relative to a dyadic combat fits the hypothesis that red males are only dominant when rare, i.e. that the dominance of red is frequency-dependent. Future studies should evaluate whether this hypothesis holds or not by studying dominance in assemblages with different proportions of red and blue males.

Learning

Learning effects on biased aggression towards intruding rival males may be important in stabilizing co-existence of nascent species during the process of speciation. Learning is theoretically an effective way of generating an own-type bias in aggression. It does not require linkage disequilibria to form between alleles causing different colors and alleles causing different aggression preferences. Two learning effects can be distinguished. First, animals could learn to preferentially attack rivals which are most frequently encountered as direct competitor for the same females or habitat. Secondly, the ancestral morph may learn to modify the level or nature of aggression towards the behaviorally dominant novel morph when the latter increases in frequency.

I tested whether learning in the adult phase affects aggression preferences (chapter 5). Laboratory-bred blue *Pundamilia* males from hybridizing- and single-species populations were either given prolonged experience with only blue (blue treatment) or both blue and red (mixed treatment). Blue-treated males had no preferences, whereas mixed-treated males had a preference for blue rival males. There was an effect of treatment, indicating a role of learning. I did not detect a
population type effect in lab-bred males, contrasting with the difference in aggression bias in their wild-caught fathers (chapter 2 & 3). This may hint at a role of learning in shaping aggression biases in the wild. Given the lack of an aggression bias in the blue treatment, it seems that males need experience with more than one morph to become selective with regards attack decisions. Finally, the direction of aggression biases in lab-bred males was not consistent with what I found in their wild-caught counterparts (chapter 2& 3). I have no explanation for this discrepancy.

I also tested the possibility that experience with red males may modulate the competitive ability of blue males in dyadic combat (chapter 4). Before a combat, some blue males were housed together with red males in a community aquarium, and others not. There was no effect of housing on the outcome of dyadic combat between blue and red males, indicating no effect of learning.

In conclusion, rearing may affect aggression biases based on nuptial coloration of the intruder, but not competitive ability in dyadic combat. Verzijden (in prep) found that _Pundamilia_ young can sexually imprint on their mother. Preferences in haplochromines can thus be obtained through learning, and are not only genetically predisposed. This is not only relevant for designing preference experiments, but also for models on speciation; many of these assume genetically controlled trait and preference (e.g. Arnegard & Kondrashov 2004; Van Doorn et al. 2004), whereas the role of learning has received less attention (Aoki et al. 2001; Verzijden et al. 2005).

**Aggression biases in females of _Neochromis omnicaeruleus_**

Territorial behavior likely affects fitness of both male and female haplochromines, especially in algae-scrapping species in which both sexes aggressively defend territories to secure the algal mat (Ribbink et al. 1983; Seehausen 1996; Maan 2006a). Female haplochromines become territorial in the mouth brooding phase to protect their fry (Seehausen 1996). If own-type biases exist in females, it could generate a ‘rare female’ advantage, and contribute to co-existence of nascent species.

The highly polymorphic cichlid _Neochromis omnicaeruleus_ represents a common female color polymorphism in haplochromines in several African lakes (see Lande et al. 2001). The species consists of three distinct color morphs, a plain morph and two blotched morphs. Choice tests revealed that female morphs of _Neochromis omnicaeruleus_ biased aggression towards their own morph (chapter 9). This may stabilize the color polymorphism in this species, because the less common morph would sustain fewer attacks, and hence enjoy elevated fitness. Why do females of _Neochromis omnicaeruleus_ bias aggression towards their own morph? In the discussion of chapter 9 I explain the own-type aggression bias in terms of competition for mates. Mutual mate choice in the _Neochromis omnicaeruleus_ system (Seehausen et al. 1999b), would place a premium on females that bias aggression towards direct competitor for potential mates.
Pleiotropy between color and aggression bias?

It is difficult to understand how own-type biases can evolve during an initial or incipient stage of speciation. Gene-flow would erode linkage disequilibrium between color and aggression bias by recombination. If own-type biases in aggression are expressed pleiotropically with color in the wake of divergent sexual selection, the co-existence of incipient species would be greatly facilitated. I tested such pleiotropic effects of color and aggression in two polymorphic haplochromine systems, *Neochromis omnicaeruleus* and the single-species population of *Pundamilia* (Luanso Island, chapter 3).

I crossed plain with blotched *Neochromis omnicaeruleus*, yielding broods containing both plain and blotched sisters. I found that own-type biases in aggression in these laboratory bred sisters were broken down, making pleiotropy or tight linkage unlikely explanations for own-type aggression biases in wild-caught females (chapter 9).

In the *Pundamilia* system, we can also test for pleiotropy/tight linkage against the alternative of independently segregating genes. We took advantage of the fact that at the single-species population (Luanso Island) males show a continuous distribution of red-blue phenotypes, although distinct red phenotypes are absent. We scored the individuals that we tested in chapter 3 using the phenotype scale in Figure 1 of chapter 3, and expressed the aggression preference for the blue morph as a function of phenotype score (see Figure 2). There was no significant relationship between aggression preference and phenotype score (Figure 2 one-sided Spearman rank correlation: \( r = -0.24, \ P > 0.1, \ N = 28 \)), making pleiotropy between color and aggression bias again unlikely.

The lack of pleiotropic color effects on aggression bias in polymorphic *Pundamilia* and *Neochromis omnicaeruleus* species suggests that a (strong) build-
up of linkage disequilibrium between color and aggression bias is required for the evolution of own-type biases in aggression. This process is less effective than pleiotropy in stabilizing speciation. Nonetheless, one would predict strong selection favoring individuals that preferentially expel competitors for mates and/or most dangerous usurpers of territorial space.

**Territory quality affects mate choice**

We need a mechanism to transmit rare male advantages in intrasexual competition to elevated fitness. One possibility is that rare males are more likely to acquire and defend a high-quality territory, and that quality cues of territories can override color-based mate choice. Therefore, I tested whether territory quality can override the female mate preference for own color in *P. nyererei* (chapter 7). Dominant males preferentially monopolize large tubes over small tubes, suggesting a high value to the former. We were able to alter the preference in favor of a heterospecific male when that male was given a large tube, suggesting that territory quality can override a preference for own color. These findings indicate that if rare male types are more readily able to obtain high-quality territories than the common male types, females could be faced with a conflict between a preference for males of own color and a preference for males with high-quality territories. If at least some females fall for the deviant-looking, but high-quality territory proprietor, rare male advantages in intrasexual competition may facilitate the propagation of their genes in the population.

The strength of the effect of territory quality on mate choice could depend on the magnitude of territory quality differences among males. To test this idea, future studies should focus on mate choice experiments in which the magnitude of territory quality differences is varied between males (see also Ritchie 1996).

That territory quality can override the female preference for own species even across genera is supported by my observation of a *Neochromis omnicaeruleus* community tank (2.5 m long), containing a single *P. nyererei* male from Python Island. I observed spawning of three *Neochromis omnicaeruleus* females with this *P. nyererei* male occupying the sole terra pot in the tank. These heterospecific matings took place despite the presence of two territorial, courting *Neochromis omnicaeruleus* males occupying sandy corners in the tank. Note that Lake Victoria haplochromines can freely hybridize resulting in fully fertile offspring (see Seehausen 2004)

**‘Good genes’ sexual selection**

The importance of female mate choice on the basis of color (Seehausen & Van Alphen 1998; Maan et al. 2004) begs the question why females should be choosy to start with. Two theories have been proposed to explain choosiness in case females accrue no direct benefits in the form of for example parental care. The first suggests that male secondary characters are purely aesthetic, signaling no inherent fitness
advantage other than attractiveness to females. Such traits are called Fisher traits (Fisher 1930). The second theory proposes that females accrue indirect genetic benefits (‘good genes’) by being choosy, resulting in offspring with increased viability and survival probabilities. Male secondary characters are under this hypothesis indicator traits, and expression is usually condition-dependent (Zahavi 1975; Rowe & Houle 1996; Jennion et al. 2001). These competing theories are also relevant in the context of intrasexual selection (see chapter 9).

Speciation theory suggests that ‘good genes’ mechanisms of female mate choice are less likely to drive (sympatric) speciation than purely divergent Fisherian runaway selection (Lande 1981; Payne & Krakauer 1997; Turner & Burrows 1995; Van Doorn et al. 1998; 2004). This is because when traits are purely Fisherian, population bifurcation may unfold in any arbitrary direction, whereas in ‘good-genes’ models of sexual selection, female mate choice is less arbitrary, directed to only those traits that reliably signal genetic quality of the male. However, empirical sexual selection research has often found evidence consistent with ‘good genes’ sexual selection (Møller & Alatalo 1999; Jennions et al. 2001; Neff & Pitcher 2005), making the assumption of purely Fisherian traits in speciation models unrealistic (Turelli et al. 2001).

In chapter 8 I asked whether ‘good genes’ sexual selection is operating in haplochromines. One test that could distinguish between Fisher and ‘good genes’ sexual selection is investigating whether males trade-off the level of sexual signaling against immunocompetence. Immune function is crucial to organisms, as they constantly need to battle parasites and pathogens. If signaling is costly, only high-quality males can afford to signal at a high level (Zahavi 1975; Hamilton & Zuk 1982). I tested this in *P. nyererei*, a haplochromine cichlid fish with a carotenoid-based nuptial dress (Maan et al. 2006c). The level of color advertisement was manipulated by social stimulation with a rival male. Social stimulation led to brighter red coloration and elevated levels of 11-ketotestosterone. Males were subsequently forced to mount a humoral immune response to a novel antigen (sheep red blood cells, SRBC). Only in the stimulated males the degree of red coloration and the antibody response were negatively correlated, suggesting a trade-off between sexual signaling and immunocompetence. This trade-off may enforce honest signaling and is consistent with the ‘good genes’ sexual selection hypothesis (Grether et al. 2003).

Androgens and/or carotenoid allocation may underlie such a trade-off (Folstad & Karter 1992; Lozano 1994). I did not detect direct immunosuppressive effects of androgens on antibody production. Adequately addressing this question requires artificial elevation of androgens levels by means of for instance hormone implants (e.g. Ros et al. 1997; Lindström et al. 2000, Peters 2000). This was attempted in *P. nyererei* and *P. Pundamilia* from Kissen Island using silastic implants filled with 11KT following Ros et al. (2004). The experiment failed in inducing required hormone levels. To resolve these issues alternative methods
should be considered, such as administration of androgens dissolved in cocoa butter by peritoneal injection (Consten et al. 2002).

Carotenoid allocation to color expression may be traded off against carotenoid availability for immunocompetence (Lozano 1994). Such evidence could come from reduced color display after immunization (Faivre et al. 2003) In chapter 9, immunization did not result in a decrease in color display. One possible explanation is that males were faced with a relatively low amount of novel antigen. Injection of *P. nyererei* with LPS (lipopolysaccharide), which causes a rapid inflammatory response (Poxton 1995), resulted in a decrease in color display, suggesting that reduced color expression may occur upon exposure to larger SRBC dosage.

Chapter 8 thus provides both correlational and experimental evidence suggesting that expression of red nuptial dress comes with an immunological cost. This may implicate that only ‘good quality’ *P. nyererei* males can afford to express color in full glory. This is consistent with but does not confirm the hypothesis of ‘good genes’ sexual selection operating in haplochromines. Further studies should concentrate on multiple components of general health and immunocompetence, such as clearance of a natural pathogen, antioxidant activity, cell-mediated immunity, etc (see e.g. Kurtz et al. 2003; 2006; Vainikka et al. 2004; 2005; Ros et al. 2006). Careful breeding experiments are required, testing whether the level of sexual signaling is related to viability in female and male offspring (e.g. Reynolds & Gross 1992; Barber et al. 2001; Hunt 2004; Neff 2004).

‘Good genes’ sexual selection is also indicated by parasite-mediated sexual selection in haplochromines (Taylor et al. 1998; Maan et al. 2006c). *P. nyererei* females prefer bright red males, and brighter males are less parasitized (Maan et al. 2004; 2006c), suggesting female mate choice against parasitized males. Given the evidence for ‘good-genes’ sexual selection in haplochromines we need a better (theoretical) understanding of the conditions under which sympatric speciation can unfold by ‘good-genes’ sexual selection.

**Concluding remarks**

This thesis provides experimental evidence supporting the hypothesis that intrasexual aggressive competition is likely to exert selection on nuptial coloration, possibly facilitating sympatric speciation.

Several mechanisms by which frequency-dependent selection can be accomplished were studied in males of *Pundamilia* and female color morphs of *Neochromis omnicaeruleus*. The work presented in this thesis is summarized in table 1. Aggression bias and dominance should be studied in isolation using simulated intruder choice tests and dyadic combats respectively. Subsequently, one should test whether the combined effect of aggression bias and dominance yields frequency-dependent dominance (table 1, last column). A first start for such an experiment is described in chapter 6. Ultimately, social hierarchies should be
studied among males or females in assemblages varying in the proportions of different morphs.

In several chapters choice tests has been employed to measure aggression preference (see table 1). Most of the reasoning starts from the premise that being a preferred target for attack leads to reduced dominance. This assumption of the work seems logic and is supported by anecdotal evidence from aquarium observations. Yet, it will require more work, and can be evaluated in a group experiment as explained above. Such an experiment is currently underway. Ultimately, rare morph advantages in intrasexual selection should translate into higher fitness. I used dominance as a proxy for fitness, which is valid considering several studies showing a relationship between dominance and reproductive success in haplochromines (Maan et al. 2004; Dijkstra unpublished)

Some broader implications

Selection on male nuptial coloration by male-male competition could also promote phenotypic divergence in reproductive characters and species co-existence in a broader sense, regardless of the phylogenetic history of species and outside the context of speciation (see e.g. Losos et al. 2003; Gillespie 2004). For example, species that are distantly related or evolved in allopatry and are subsequently brought into sympathy can undergo character displacement as a result of interspecific aggressive interactions (see also Alatalo 1994; Seehausen & Schluter 2004; Tynkkynen et al. 2004; 2006). In addition, similarity in nuptial dress between resident and immigrant species may determine success or failure of range expansion of the latter.

<table>
<thead>
<tr>
<th>Population type</th>
<th>aggression bias</th>
<th>dominance</th>
<th>frequency-dependent dominance</th>
</tr>
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<tbody>
<tr>
<td>Pundamilia</td>
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<td></td>
<td></td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
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<td>0</td>
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</tbody>
</table>

*¹ This work has been done, but has not yet been described in a manuscript: I found dominance of white blotched females over orange blotched and plain females, and indications of pleiotropy between colour and dominance.
Speciation research in haplochromines has been polarized between sexual and natural selection (e.g., Arnegard & Kondrashov 2004). However, male-male competition hypothesis is not only relevant for speciation by sexual selection, but also for ecological speciation: it may stabilize co-existence of nascent species, allowing time for the build-up of ecological differences.

It seems likely that natural and sexual selection act in concert (see also Maan 2006). First, color morphs or sister species often show subtle spatial segregation or variation in morph frequency, either along a vertical axis or across a heterogeneous habitat consisting of for instance rocky substrate interspersed with small sandy patches. For example, *P. nyererei* usually occurs deeper than *P. Pundamilia; P. ‘pink anal’* also occupies sandy patches, whereas syntopically occurring *P. nyererei* solely occupies rocky territories.

Several forces shape the evolution of male color patterns: female mate choice as well as male-male competition is likely to select for bright conspicuous males, with predation as a counterveiling force (Maan 2006). Conspicuousness, in turn, is determined by intensity and spectral composition of the ambient light, by the color contrast with the background color, and on the visual properties of the observer (Endler 1978, see also Seehausen et al. 1997; Fuller 2002; Maan 2006; Maan et al. 2006b). Light conditions vary with depth in Lake Victoria, with longer wave length penetrating deeper, and favoring the evolution of more reddish coloration. The role of ambient light and color vision is discussed at length in Maan (2006). Maan (2006) concludes that selection pressures driving the evolution of haplochromine color patterns are habitat-dependent. In conjunction with ‘good genes’ sexual selection, this may create differences in trade-offs between habitats, allowing females to select for males with a color that signals adaptation to the local environment, and to recognize and avoid maladapted males. In other words, females can then select for locally adapted males that can produce sexual signals of superior attractiveness. Such a scenario of ‘good genes’ sexual selection for locally adapted males has recently been indicated to be a plausible scenario of sympatric speciation (Edelaar et al. 2004; Reinhold 2004; see also Day 2000; Proulx 2001).

Lake Victoria haplochromines are not only famous for their staggering explosive speciation, but also for the rapid loss of hundreds of unique species. This was the result of the immense species extermination due to explosion of the introduced Nile perch in the early 80s (Witte et al. 1992; Goldschmidt 1994), and more recently, increased eutrophication and polution, associated loss of visibility and relaxation of sexual selection (Seehausen et al. 1997; Seehausen 2006b). I hope my thesis will contribute to a better understanding of the biology of haplochromine cichlid fish, drawing some attention to their unique species radiation and hence need of conservation of their ecosystem.
Nederlandse samenvatting

Intraseksuele selectie en sympatrische soortvorming bij Victoria cichliden

Peter D. Dijkstra
Soortvorming

Hoe kan uit één diersoort nieuwe diersoorten ontstaan? Binnen de evolutiebiologie probeert men de processen te ontrafelen die soortvorming veroorzaken. Begrip over soortvorming is niet alleen van belang voor fundamentele vragen over het ontstaan van biodiversiteit, maar ook voor het verschaffen van informatie voor natuurbeschermers. Tevens is het belangrijk biologische systemen te bestuderen voordat we ze wellicht verliezen door menselijk toedoen.

Klassieke studies van soortvorming benadrukten het belang van geografische scheiding: geografisch geïsoleerde populaties kunnen geen genen meer met elkaar uitwisselen, en bewandelen hierdoor ieder hun eigen evolutionaire weg. Na verloop van tijd kunnen ze van elkaar verschillen. Wanneer ze weer met elkaar in contact komen zullen ze niet meer met elkaar mengen omdat ze elkaar bijvoorbeeld niet meer als soortgenoten herkennen. Op dat moment zijn er twee nieuwe soorten ontstaan (allopatrische soortvorming).

Geleidelijk heeft het idee aan kracht gewonnen dat soorten ook zonder die geografische barrières (zoals een bergketen of rivier), en dus met mogelijkheden voor uitwisseling van genen, kunnen ontstaan. We noemen dit sympatrische soortvorming. In deze situatie is soortvorming een veel ingewikkelder proces. Hoe is het mogelijk dat populaties uit elkaar evolueren als ze elkaar voortdurend tegen het lijf lopen en genen kunnen uitwisselen? Het sympatrische soortvormingmodel is dan ook decenniaal lang controversieel geweest (Schilthuizen 2001). De laatste jaren is er echter een redelijk consensus ontstaan over de mogelijkheid dat sympatrische soortvorming mogelijk is wanneer het gedreven wordt door ecologische selectie. Stel een populatie voor waarbij een aantal dieren zich gaat specialiseren op kleine prooidieren, en anderen op grote prooidieren. Er kan hierdoor een klein verschil of divergen tie ontstaan in lichaamsgrootte. Deze subpopulaties kunnen van elkaar reproductief gescheiden raken als er een vrouwelijk voorkeur ontstaan voor het kenmerk wat gekoppeld is aan de ecologische specialisatie, in dit geval lichaamsgrootte. Bij dit proces is ecologische selectie de drijvende kracht achter de soortvorming, en speelt de seksuele voorkeur van vrouwtjes, of seksuele selectie, een secundaire rol.

Sympatrische soortvorming: Het ontstaan van dochtersoorten uit een vooroudersoort, zonder dat er een geografische barrière, zoals een rivier, bergketen of zee, tussen de toekomstige dochtersoorten zit. Het tegenovergestelde is 'allopatrische soortvorming' waarbij die barrière er wel is en de toekomstige dochtersoorten dus op een eenvoudige en duidelijke manier gescheiden zijn.

Sexuele selectie: een proces waarbij bepaalde eigenschappen van een dier het makkelijker maken dat deze een partner krijgt, waardoor deze meer nakomelingen krijgt.

Ecologische selectie: een proces waarbij organismen die beter zijn aangepast aan de ecologische omstandigheden meer nakomelingen krijgen dan minder aangepaste organismen.
Omdat soortvorming doorgaans erg lang duurt is het een moeilijk te bestuderen proces. We hebben een groep dieren nodig die extreem snel evolueren, zodat we het soortvormingproces in verschillende stadia onder de loep kunnen nemen. Een excellent modellsysteem zijn de spectaculaire soortenzwermen van snelevoluerende haplochromide cichlide vissen uit de Oost-Afrikaanse meren.

Soortvorming binnen haplochromine cichliden


Een grote hoeveelheid haplochromine soorten in het Victoriameer zijn in de afgelopen decennia uitgeroeid door de geïntroduceerde Nijlbaars (Goldschmidt 1994). Gelukkig zijn de gevolgen van de Nijlbaars minder rampzalig voor de kleurrijke rotscichlidengemeenschap in het Victoriameer. Mijn proefschrift richt zich op deze cichliden.

De cichliden uit het Victoriameer broeden de eieren uit in de bek. Normaal gesproken zijn alleen de mannetjes felgekleurd. Mannetjes strijden driftig om de schaarse territoria en proberen met zoveel mogelijk vrouwtjes te paren. Na de
bevruchting draait alleen de vrouw op voor de broedzorg. Deze oneerlijke verdeling van zorgtaken maakt vrouwtjes kieskeurig bij het uitzoeken van een mannetje; waarschijnlijk wil ze paren met het mannetje met de juiste genetisch bagage voor het nageslacht. Die kieskeurigheid, oftewel seksuele selectie op de kwaliteit van de mannetjes, heeft gevolgen voor het ontstaan en voortbestaan van soorten. De vrouwelijke voorkeur voor specifieke mannen zou het uiterlijk van mannen kunnen veranderen, en daarmee nieuwe soorten doen ontstaan.

De rol van intraseksuele selectie bij soortvorming

Het vermoeden bestaat dat veel cichliden zijn ontstaan zonder geografische barrières. In de voorgaande paragraaf heb ik al uiteengezet dat in het orthodoxe sympatrische soortvormingmodel ecologische selectie een sleutelrol speelt, en dat seksuele selectie van ondergeschikt belang is. Bij de haplochromine cichliden is echter iets interessants aan de hand: veel kleurvarianten en zustersoorten verschillen dramatische in de kleur van het mannelijke broedkleed, en nagenoeg niet in morphologische aanpassingen voor bijvoorbeeld specifiek eetgedrag. Is het wellicht mogelijk dat sympathische soortvorming in de haplochromine cichliden gedreven wordt primair door seksuele selectie? Kan variatie in vrouwelijk voorkeur voor specifieke mannen de evolutie van verschillende kleurvarianten- en soorten bewerkstelligen? Zou de vrouwelijke kieskeurigheid de rol van een geografische barrière kunnen overnemen, en ervoor kunnen zorgen dat subpopulaties minder genen gaan uitwisselen en op den duur uit elkaar evolueren?

Deze theorie is nog steeds erg controversieel. Allereerst is het lastig voor een nieuwe kleurvariant om een populatie binnen te dringen. De meeste vrouwtjes hebben immers een voorkeur voor de reeds bestaande kleurvariant, en de nieuwe kleurvariant heeft daarom een extra duw in de rug nodig om een poot aan de grond te krijgen. Het tweede probleem is het stabiel voorkomen van twee kleurvarianten: als deze kleurvarianten zich niet ecologisch gaan onderscheiden wordt de concurrentie om ruimte en voedsel normaliter een van beiden fataal. Met andere woorden: hoe kan kleurvariatie in een populatie ontstaan en worden gehandhaafd? We denken dat deze problemen verholpen kunnen worden als afwijkende kleurvarianten een voordeel hebben bij agressieve interacties met andere mannetjes doordat ze bijvoorbeeld niet herkend worden als rivaal. Als zeldzame mannetjes een hogere kans hebben een mooi territorium te bemachtigen zouden ze wellicht een vrouwtje kunnen verleiden, ook al zien ze er afwijkend uit. Zo’n voordeel bij man-man competitie, ofwel intraseksuele selectie, noemen we het ‘zeldzame mannetjes’ effect. De term ‘zeldzaam’ geeft al aan dat dit voordeel minder wordt als de nieuwe kleurvariant in frequentie toeneemt, en dus minder zeldzaam wordt. Met andere woorden, we denken dat de strijd tussen de mannen negatief frequentie-afhankelijke selectie op het broedkleed kan opleveren. Frequentie-afhankelijk selectie zorgt ervoor dat twee kleurvarianten alleen een voordeel hebben als ze zeldzaam. Hierdoor kunnen kleurvarianten stabiel samen voorkomen, waaruit vervolgens door vrouwelijke kieskeurigheid verschillende
soorten kunnen evolueren. Voor de uiteindelijk soortvorming blijft seksuele selectie door vrouwelijk voorkeur op mannelijke kleurpatronen nog steeds erg belangrijk is. Kleur en de corresponderende vrouwelijke voorkeur moeten gekoppeld raken, waardoor restrictie in genenuitwisseling tussen subpopulaties kan ontstaan.

De studie van seksuele selectie en soortvorming richt zich traditioneel hoofdzakelijk op de vrouwelijke voorkeur voor mannelijke kleur patronen (interseksuele selectie). De effecten van competitie tussen mannetjes op de evolutie van kleur patronen en vrouwelijke voorkeuren (intra seksuele selectie) is pas recent onder de aandacht gekomen. Theoretisch werk van Sander van Doorn (2004) laat zien dat juist een combinatie van inter- en intra seksuele selectie essentieel is voor sympatrische soortvorming.

Hoe kan man-man competitie een voordeel opleveren voor zeldzame mannetjes? Zeldzame mannen hebben een voordeel als ze bijvoorbeeld minder agressie ontvangen of een verhoogde kans hebben conflicten te winnen. Dit kan op verschillende manieren bewerkstelligd worden.

(1) Agressievoorkeur voor de eigen kleur
Als mannetjes agressiever zijn naar rivalen met hun eigen kleur dan een andere kleur, hebben afwijkend gekleurde mannetjes automatisch een voordeel: ze worden dan door de meute niet herkend als rivaal. Hierdoor hebben ze minder last van aanvallen, maar krijgen het moeilijker als hun eigen kleurvariant in frequentie toeneemt omdat ze dan steeds meer aanvallen te verduren krijgen van kleurgenoten.

(2) Dominantie door kleureffecten
Kleuren als rood of oranje kunnen door hun intimiderende uitstraling de kans vergroten dat de drager een conflict wint. Als de nieuwe zeldzame kleurvariant zo’n kleur heeft zou hem dat een voordeel kunnen opleveren in de competitie voor territoria.

(3) Dominantie door een hoger agressieniveau
Als de nieuwe zeldzame kleur gekoppeld is aan een hoger agressieniveau zou dat een voordeel kunnen opleveren in conflictsituaties.

De laatste twee mechanismen vergen een extra aannames voor frequentieafhankelijkheid, namelijk dat de dominante kleurvariant aan vechterkracht inboet zodra deze in relatieve frequentie toeneemt, doordat ze bijvoorbeeld elkaar in onderlinge gevechten meer uitputten dan de andere kleurvariant.

Mijn proefschrift beschrijft een reeks aan experimenten waarin assumpties en voorspellingen van de ‘zeldzame man’ hypothese zijn getest.
De resultaten

De hoofdrolspelers in mijn proefschrift zijn *Pundamilia Pundamilia* (blauw) en *Pundamilia nyererei* (rood), twee nauw verwante soorten die voornamelijk in mannelijk broedkleed van elkaar verschillen. Deze twee soorten vertegenwoordigen een zeer algemeen voorkomende kleurvariatie tussen zustersoorten in het Victoriameer. Omdat rood altijd gemengd voorkomt met blauw terwijl blauw wel ongemengd voorkomt, denken we dat blauw de voorouderlijke soort is, en dat rood herhaaldelijk blauwe populatie is binnengedrongen.

Agressievevoorkeuren

We hebben drie type populaties onderzocht: (1) een populatie waarin alleen blauw voorkomt (blauwe populatie), (2) twee populaties waar rood al is binnen gedrongen maar waar rood en blauw nog steeds genen uitwisselen (hybridiserende populaties), en (3) drie populaties waarbij rood en blauw echte soorten zijn, dus met totaal geen uitwisseling van genen (gescheiden populaties). Deze drie populatietypen zou men als een gradiënt van soortvorming kunnen beschouwen: van een maagdelijke populatie, naar de vestiging van een nieuwe kleurvariant, tot het ontstaan van twee reproductief gescheiden, dus ‘echte’ soorten.

In onze zogenaamde agressiekeuzetest boden wij een territoriale man twee verschillend gekleurde rivalen aan in doorzichtige buizen, zowel in aquaria als in het veld (Tanzania). We maten dan de agressie van een mannetje richting de twee rivalen en konden zo zijn voorkeur voor een beide gekleurde rivalen bepalen. Hoofdstuk 2 en 3 laat zien dat mannetjes uit het wild inderdaad meer agressie richten naar rivalen van hun eigen kleur. Echter, dit is alleen het geval bij blauwe populaties en gescheiden populaties; in hybridiserende populaties richten blauwe mannen meer agressie naar rood. Dit duidt erop dat agressie voorkeuren de invasie van rood zouden kunnen begunstigen, alsmede het stabiel samen voorkomen van reproductief gescheiden zustersoorten. Dat komt doordat in de eerste situatie rood niet als rivaal herkend. In de laatste situatie ontvangen zowel rood als blauw minder agressie als ze zeldzaam zijn. Echter, als het soortvormingsproces nog in gang is, zoals dat het geval is in de hybridiserende populatie, dan zijn agressievevoorkeuren niet voldoende om het proces te stabiliseren omdat rood dan altijd meer agressie ontvangt. De conclusie moet dan ook zijn dat agressievevoorkeuren op zichzelf soortvorming niet kunnen stabiliseren. Echter, ik denk dat agressievevoorkeuren in combinatie met andere elementen van man-man competitie, zoals dominantievoordelen door kleur, wel de vereiste frequentie-afhankelijkheid kan generen. Zo laat ik in hoofdstuk 4 zien dat rood dominant is over blauw (zie volgende sectie). Het is wellicht mogelijk dat een combinatie van agressievevoorkeuren en dominantie van rood soortvorming in hybridiserende populatie kan stabiliseren. Tenslotte laat ik in hoofdstuk 2 zien dat
agressievevoorkeuren gebaseerd zijn op de kleuren van het broedkleed: is het onderscheid van beide kleuren niet goed zichtbaar (dit kan gecreëerd worden met groen licht), dan maken de vissen ook geen onderscheid tussen verschillend gekleurde rivalen.

Hoe ontwikkelen mannetjes hun agressievevoorkeuren? Is het voornamelijk genetische aanleg, of komt er ook nog leren aan te pas? Het is bijvoorbeeld mogelijk dat mannetjes van alle populatietypen een voorkeur vertonen om de eigen kleurvariant aan te vallen, maar dat blauwe mannetjes in de hybridiserende populatie hebben geleerd meer aandacht aan rode mannetjes te schenken omdat ze gevaarlijke, agressieve rivalen zijn. In hoofdstuk 5 testen we de hypothese dat ervaring met alleen maar blauw leidt tot een voorkeur voor blauw, en dat ervaring met zowel rood als blauw leidt tot een voorkeur voor rood. In deze studie gebruikten we labkweek van blauwe mannetjes uit de blauwe populatie en twee hybridiserende populaties. De resultaten gingen tegen onze verwachting in. Mannetjes met uitsluitend ervaring met blauw hadden geen agressievevoorkeur, terwijl mannetjes met ervaring met beide kleurvarianten een voorkeur hadden om blauw aan te vallen. Ook al kunnen we deze resultaten moeilijk interpreteren, ze laten in ieder geval zien dat agressievevoorkeuren niet alleen genetische zijn geprogrammeerd, maar ook gevormd worden door leereffecten.

Dominantie

Hoofdstuk 4 en 6 richten zich op labkweek van blauwe en rode mannetjes uit een hybridiserende populatie.

In hoofdstuk 4 heb ik rode en blauwe mannetjes één-op-één tegen elkaar laten vechten. We zagen dat rode mannetjes veel meer conflict wisten dan blauwe, maar dit voordeel verdwijnt als het kleurverschil tussen de vechtersbazen wordt gemaskeerd met groen licht. Blijkbaar heeft rood een intimidend effect op blauwe rivalen en kan dit kleureffect wellicht de invasie van rode kleurvarianten in een blauwe populatie vergemakkelijken.

In hoofdstuk 6 worden eerdere bevindingen onder meer natuurlijk omstandigheden gevalideerd. Groepjes van alleen rode of alleen blauwe mannetjes (pure groepen), alsmede groepjes met dezelfde aantallen van beide kleurvarianten (gemengde groepen) werden bestudeerd in grote aquaria. Ik stelde de volgende vragen: (1) Meten we in deze groepjes dezelfde agressievevoorkeur als in een agressiekeuze experiment?; (2) Rood is in een één-op-één conflict dominant over blauw, maar in het veld zien we nooit dat rood blauw verdringt, er bestaan immers geen pure rode populaties. Is de dominantie van rood gelijk aan die van blauw in een gemengde situatie, in tegenstelling tot de één-op-één situatie? En tenslotte (3): de ‘zeldzame man’ hypothese voorspelt dat het agressieniveau in pure groepen hoger is dan in gemengde groepen. Dat komt omdat in een gemengde situatie mannetjes andersgekleurde rivalen kunnen ontmoeten die minder agressie opwekken dan rivalen van de eigen kleur. Is het dan zo dat qua agressieniveau en de kosten van territoriale verdediging een gemengde groep voordeliger en stabieler

**Territoriumkwaliteit en partnerkeuze**

Een voorwaarde voor het ‘zeldzame man’ effect is dat voordelen in man-man competitie zich vertalen in een verhoogd reproductief succes. Mannetjes concurreren driftig om territoria die variëren in grote en structurele complexiteit. Stel je voor dat zeldzame mannen een hogere kans hebben een mooi territorium te bemachtigen. Zou territoriumkwaliteit er dan voor kunnen zorgen dat zo’n afwijkend gekleurd mannetje toch aan de vrouw komt? In hoofdstuk 7 laat ik zien dat mannetjes bij voorkeur een grote tube als centrum van hun territorium bezetten. Vervolgens hebben we een rood vrouwtje laten kiezen tussen een blauwe en een rode partner. Zoals verwacht bleken rode vrouwjes een seksuele voorkeur te hebben voor een rode man. Echter, zodra we de blauwe man een grote tube gaven en de rode de minder aantrekkelijke kleine tube vertoonde de vrouwjes geen voorkeur meer voor één van de twee mannetjes. Blijkbaar kan territoriumkwaliteit de voorkeur voor de eigengekleurde man teniet doen. Als zeldzame mannen een grotere kans hebben een hoogwaardig territorium te bezetten, dan zouden vrouwjes wellicht met een dilemma geconfronteerd worden tussen voorkeur voor de eigen kleur en voorkeur voor mannetjes met een mooi territorium. Dit effect zou de vestiging van een nieuwe kleurvariant kunnen faciliteren.

**Kleuren en kwaliteit**

In veel diersoorten vertonen mannen veel variatie in de intensiteit van het broedkleed. Dat is ook sterk het geval in onze rode cichlide, *P. nyererei*. Werk van Martine Maan uit Leiden aan dezelfde soort heeft laten zien dat vrouwjes een voorkeur hebben voor felgekleurde kerels (Maan 2006). Misschien is het voordeel van kieskeurigheid erfelijk: een felgekleurde man kan voor felgekleurd nageslacht zorgen die dan ook weer goed in de markt ligt. De vraag is nu: gaan vrouwjes puur
vanwege esthetisch redenen af op felgekleurde mannetjes (‘sexy sons’ hypothese), of verschaf de kleurintensiteit informatie over de genetisch kwaliteit van de drager die de overleving van het nageslacht kan vergroten, bijvoorbeeld resistentie tegen parasieten (‘good genes’ hypothese)? Een belangrijke assumptie in de theorie over sympatrische soortvorming is dat seksuele selectie via het ‘sexy sons’ mechanisme verloopt, omdat dan de evolutie van de kleur van het broedkleed veel willekeuriger is dan onder ‘good genes’ seksuele selectie. Hierdoor kan soortvorming makkelijker plaatsvinden. Echter, in de literatuur over seksuele selectie persé wordt veelal juist uitgegaan van de ‘good genes’ hypothese.

Als de intensiteit van het broedkleed iets zegt over de genetische kwaliteit van de drager (‘good genes’) dan zou kleurexpressie kostbaar voor het mannetje moeten zijn. Dit voorkomt dat mannetjes oneerlijke informatie kunnen geven door via kleur te bluffen over hun kwaliteit. Kleurexpressie zou bijvoorbeeld ten koste kunnen gaan van het functioneren van het afweersysteem, waardoor alleen individuen met een van nature heel goed afweersysteem zich felle kleuren kunnen veroorloven. Wij testten dit in de soort *P. nyererei* (hoofdstuk 8). Allereerst bleek dat sociale stimulatie met een rivaal leidde tot felle kleurexpressie. Vervolgens werden de mannen blootgesteld aan een lichaamsvreemd eiwit waartegen ze antilichamen gaan maken. Het bleek dat extreem rode mannen een lagere antibodyproductie hadden dan minder roodgekleurde mannen. Dit duidt erop dat kleurexpressie ten koste gaat van het functioneren van het afweersysteem, en ondersteunt de hypothese dat de rode kleur in *P. nyererei* een signaal is over de kwaliteit van de drager. Zoals we al weten heeft de kleur van het mannelijk broedkleed een belangrijke rol gespeeld bij de explosieve soortvorming van Victoriacichliden. De discrepantie tussen ‘good genes’ sexuele selectie en de verminderde kans op soortvorming noopt om een frisse, herziene blik op het soortvormingproces bij haplochromide cichliden. In de conclusie kom ik hier nog op terug.

*Zeldzame vrouw effect*

Conclusies

In mijn laatste hoofdstuk, de synthese, vat ik de resultaten van mijn proefschrift samen, en bespreek deze in hun onderlinge samenhang. Ik laat zien dat kleur belangrijk is bij de competitie tussen mannen (hoofdstuk 2, 4 & 6). Behalve dus dat kleur onderhevig is aan seksuele selectie door vrouwelijke voorkeuren, staat het ook onder intraseksuele selectiedruk. Dat competitie tussen mannetjes erg belangrijk is bij seksuele selectie wordt ook ondersteund door hoofdstuk 7 waarin beschreven wordt dat territoriumkwaliteit erg belangrijk zou kunnen zijn bij partnerkeuze.

In de introductie beschreef ik al dat frequentie-afhankelijke selectie op verschillende manieren bewerkstelligd kan worden. Mijn proefschrift geeft aan dat intraseksuele selectie inderdaad is opgebouwd uit verschillende elementen, zoals agressievoorkeuren, kleur effecten op dominantie en asymmetrie in agressieniveau tussen kleurvarianten of zustersoorten. Hoe dit wel, dan niet frequentieafhankelijke intraseksuele selectie kan opleveren zou bestudeerd kunnen worden zoals in hoofdstuk 6. Men zou groepen van blauwe en rode mannen kunnen bestuderen met verschillende proporties van beide kleuren, en dan kunnen kijken naar frequentie-afhankelijke dominantie: als rood zeldzaam is heeft deze meer kans om dominant te worden, en vice versa.

Een belangrijke conclusie van mijn proefschrift is dat man-man, en ook vrouw-vrouw competitie een belangrijke kracht is bij het ontstaan en gehandhaafd blijven van nieuwe kleurvarianten en soorten. Mijn proefschrift laat zien dat intraseksuele selectie de vereiste frequentie-afhankelijkheid zou kunnen opleveren die infiltratie van nieuwe kleurvarianten, alsmede het stabiel voorkomen van meerdere kleurvarianten zou kunnen stabiliseren.

Ik maak aannemelijk dat de kleur informatie zou kunnen verschaffen over de genetische kwaliteit van het mannetje. In theorie maakt dit de ontwikkeling van kleur minder willekeurig, waardoor soortvorming door seksuele selectie minder makkelijk kan optreden. Edoch, in de laatste paragraaf schets ik een genuanceerder beeld van soortvorming gedreven door seksuele selectie alleen. Ik benadruk dat seksuele en ecologische selectie hand in hand kunnen gaan. Allereerst is er variatie in habitat type (bijvoorbeeld rots versus zand; diep versus ondiep) dat effect heeft op de evolutie van kleurvormen: kleuren kunnen per habitat verschillen waar ze maximaal tot hun recht komen in termen van opvallendheid of felheid. Dit kan komen door contrast tussen broedkleed en habitat, of door kleurfiltering in de waterkolom (Maan 2006). Recent is er meer aandacht ontstaan voor sympatrische soortvorming door habitat-afhankelijke ‘good genes’ seksuele selectie (zie ook Van Doorn 2004; Maan 2006).

Mijn proefschrift laat zien dat bij het ontstaan en behoud van haplochromine soorten in het Victoriameer kleur niet alleen belangrijk is voor seksuele selectie door vrouwtjes, maar ook voor de concurrentie van mannetjes onderling. De zichtbaarheid van deze kleurpatronen, en dus de helderheid van het
water zijn van cruciaal belang voor het behoud van de haplochromine cichliden uit het Victoriameer. In dit opzicht onderstreept het proefschrift de boodschap van natuurbeschermers: om de kleuren- en soortenrijkdom van de haplochromine cichliden te behouden mag het meer niet troebeler en smeriger worden.
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Dankwoord

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