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Origin, maintenance and evolutionary consequences of male mating preference variation in East African cichlid fishes

Michele ER Pierotti

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Department of Biological Sciences

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CONTENTS

This thesis is the result of work carried out by the candidate only, with the exception of the following collaborations: the compositional data analysis of male mating preferences in chapter II was developed together by Josep Martin Fernandez (University of Girona) and myself, during my visit at the Departament d'Informatica i Matematica Applicada of the University of Girona. The preliminary experiment in chapter IV was run by Nicola Barson and Mairi Knight. In the same chapter, the statistical analysis in R software was developed by Simone Immler and myself.

All experimental procedures used in the course of this Phd thesis adhered to the Association for the Study of Animal Behaviour/Animal Behavior Society Guidelines for the Use of Animals in Research and were conducted in compliance with the United Kingdom Home Office Guidance on the operation of the UK Animals (Scientific Procedures) Act 1986.

PROLOGUE AND ROAD MAP

This thesis is about sex, colour and preferences. The explosive adaptive radiation of haplochromine cichlids in the East African Lakes has puzzled researchers well before the architects of the Modern Synthesis had led the foundations of a scientific investigation on the mechanisms of speciation (Plate 1913; Regan, 1920, 1921, 1922). Both Mayr (1942) and Huxley (1940) favoured a micro-allopatric speciation scenario coupled with multiple invasions from neighbouring river systems, though there was no lack of dissent (Kosswig, 1947; Baerends & Baerends-van Roon, 1950) in the scientific community.

Interestingly, the possibility of sympatric speciation within the East African Lakes has been very often associated with sexual selection as a facilitating or even driving force (Kosswig, 1947; Fryer & Iles, 1972; Dominey, 1984; Seehausen *et al.*, 1997; Seehausen *et al.*, 1999).

A burst of theoretical studies have recently tackled the role of sexual selection in causing sympatric speciation (Turelli *et al.*, 2001) and new empirical evidence is rapidly accumulating (Panhuis *et al.*, 2001).

Seehausen *et al.* (1997) and Lande *et al.* (2001) envisage two evolutionary pathways that might explain the extraordinary rates of speciation of the Lake Victoria and Malawi haplochromine cichlids. The first is based on variation in the perception of sexual signals by the choosy sex. Differences in sensitivity will be reflected in a polymorphism in mate preferences that, in turn, can lead to the origin of a polymorphism in signal emission in the other sex. Disruptive sexual selection on signal polymorphism has the potential to drive rapid sympatric speciation (Seehausen *et al.* 1997).

A different mechanism is associated with a widespread male and female polymorphism in colour and sex determination (Seehausen *et al.* 1999; Lande *et al.* 2001). Here, an X-linked female determiner is physically linked to a colour gene that confers a characteristic blotched pattern. An autosomal recessive male determiner acts as a male rescue factor, therefore matings between females possessing the female determiner and males carrying its autosomal suppressor, give even sex ratios in the offspring.

Sex ratio selection resulting from the invasion of the female determiner selects for male and female mating preferences, thanks to its linkage with colour genes, resulting in disruptive sexual selection and sympatric divergence, in the absence of geographical isolation or ecological differentiation.

The aim of this PhD project is to investigate the nature and effects of male mating preferences in driving divergence in sex and colour polymorphic species of Lake Victoria and Malawi haplochromine cichlids, and to test crucial assumptions and the generality of Lande *et al.* (2001) model of rapid speciation by sex reversal and sexual selection.

CHAPTER I introduces the main ingredients of this complex sex factor and colour polymorphism. The first part of the chapter addresses sex determination (part I). Describing the origin and degeneration of sex chromosomes, the rise of new sex determiners and the proximate mechanisms facilitating such takeovers, I will try to suggest that the apparent stasis of the sex determination pathway is only a misconception deriving from the apparent stability of our mammalian sex chromosome system. On the contrary, new master sex determiners can invade a population and rapidly override the ancestral sex determination system, their appearance being an almost inevitable consequence of sex itself. Reversals of heterogametic system might then be relatively frequent.

Different sex factors can be present in different populations of the same species and multiple sex factors can sometimes coexist in the same population. There is some evidence that both conditions are present in blotch colour polymorphic cichlids of the East African lakes, the focus of this thesis. These species share a W-linked colour pattern of black blotches on a white, yellow, or orange background.

Sex linkage of a trait (part II) can have profound consequences on the maintenance of the trait itself and sometimes on the sex factors to which it is linked, too. Some traits are expected to be sex-linked as they are beneficial to one sex but deleterious to the opposite sex, i.e. when sexually antagonistic. I discuss some of the consequences of sex linkage of sexually antagonistic traits as well as sexually selected traits and mating preferences on the evolution of sex determination. I propose that the accumulation of sexually antagonistic genes on the sex chromosomes can oppose or favour sex reversal. On the other hand, sex reversals might change the likelihood for the evolution of sexual selection by good genes or Fisherian runaway.

W-linkage confines the trait to females opening the possibility for the evolution of male mating preferences for female-limited colour patterns. I present the evidence for female ornamentation and the theoretical conditions for the evolution of male and mutual mating preferences (part III).

Finally (part IV), variation in sex determining factors, sex linkage of colour genes and male and female mating preferences for sex-linked colour patterns all come together in the complex genetic system of *Neochromis omnicaeruleus* and other blotch polymorphic species from Lake Victoria and Lake Malawi. I present Seehausen et al.'s (1999) experimental study on *Neochromis omnicaeruleus* and I then discuss a model by Lande et al. (2001) that, taking inspiration from Seehausen et al.'s (1999) work, finds conditions for rapid speciation by the combined action of selection on sex determining genes and sexual selection.

In CHAPTER II, a wild sample of *Neochromis omnicaeruleus* males from the colour polymorphic population of Makobe Island, is tested for the presence of male mating preferences for female colouration. I find large variation in the strength and direction of male mating preferences at least partly consistent with theoretical predictions on the

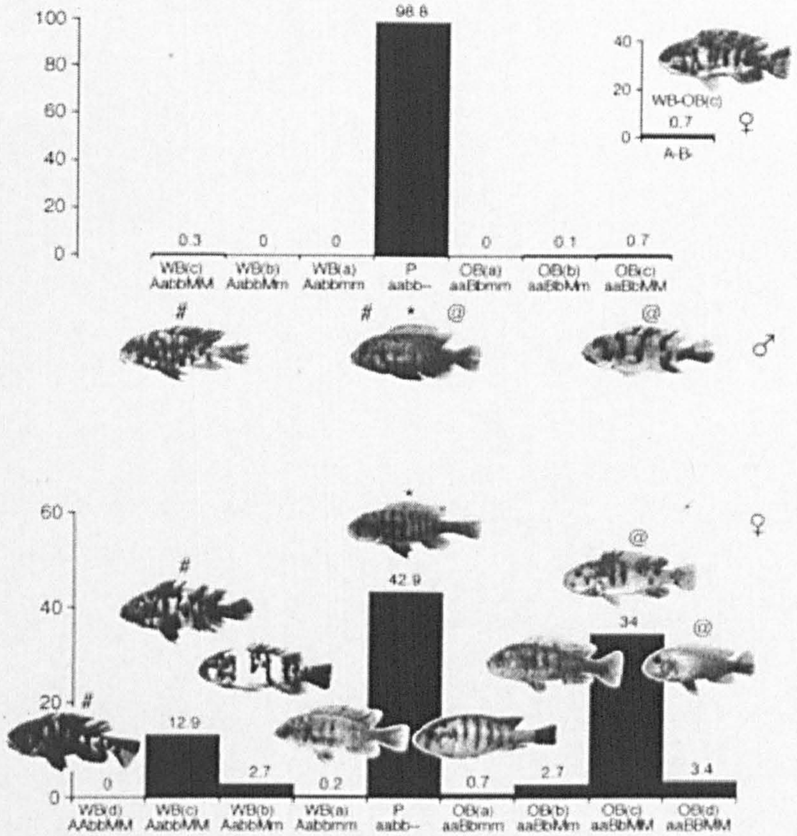

Figure 1. The colour polymorphism in *N. omnicaeruleus*. Figure legend from Seehausen et al. (1999): *Phenotypes, genotypes and natural frequencies of the phenotypes (top, males; bottom, females). P, plain; WB, black and white blotched; OB, orange blotched (WB and OB are female determiners). WB-OB, intermediate between WB and OB. A, a, alleles at x-linked WB blotch locus; B, b, alleles at x-linked OB blotch locus; M, m, alleles at autosomal locus (M represents two recessive male rescue alleles and additive blotch expressivity modifiers M_A and M_B, see text). The three common P phenotypes in both sexes are those from P parents, WB(c) mothers and OB(c) mothers. They differ at the autosomal locus and in mating preferences. Female and male morphs with mutually compatible mating preferences are designated by symbols [*, morphs of plain original species; #, morphs of black and white blotch (WB) incipient species; @, morphs of orange blotch (OB) incipient species]. Information on mating preferences within and between the original species and the WB incipient species is based on Seehausen et al.'s (1999) mate choice experiments.*

That preferences within and between the original species and the OB incipient species parallel those in the WB system is an assumption supported by experiments with a wild OB(c) female that preferred blue over WB males but did not discriminate between a blue and an OB male (unpublished), and by field underwater observations.

evolution of mating preferences for genotype matching under sex ratio selection. However, most individuals (the complete dataset when a single generation - 2003 collection - is considered) showed preference bias against non-blotched females. This suggests that at present both OB and WB females might have higher fitness than P females and that this difference is larger than the costs associated with sex ratio distortion in offspring from blotched mothers and possibly YY sons' inviability.

It is also possible that spreading of male rescue genes in response to a relaxation of selection on blotched males might reduce the costs associated with mating with blotched females and that mate choice for compatibility (sex ratio) genes is now being converted, through environmental change, into mate choice for good genes.

In CHAPTER III, a fundamental assumption of Lande et al.'s (2001) models of sympatric speciation by sex reversal and sexual selection, is tested. In particular, the dynamics leading to within-population male mating preference polymorphism requires the pre-existence of male mating preferences against a trait at the time not present in the population. I considered a monomorphic population of *Neochromis omnicaeruleus* where the P morph is present, only. I tested whether males have mating preferences for female colouration in a population fixed for one female colour morph and whether these preferences originated by reinforcement or reproductive character displacement with sympatric species. I find strong male mating preferences against blotched conspecifics. When tested with a blotched conspecific and a non-blotched heterospecific of a sympatric species, *N. omnicaeruleus* males preferred the heterospecific suggesting that reinforcement is an unlikely explanation for the evolution of male preferences and rather a pre-existing mating preference possibly evolved in ancestral species. These results show that Lande et al.'s (2001) crucial assumptions are biologically sound and that monomorphic populations like the one tested might have the potential to evolve rapidly male preference polymorphism when a novel trait such as blotched colouration invades either by mutation or by introgression with other species.

CHAPTER IV addresses the issue of the mode of inheritance of male mating preferences and at the same time tests whether the presence of a blotched colour polymorphism in a totally distinct cichlid radiation (i.e. Lake Malawi superflock) has generated male mating preferences for female colour morphs, in analogy with the pattern found in Lake Victoria blotch polymorphic cichlids. I find extreme variation in strength and direction of individual male mating preferences for female colour morphs in the Lake Malawi cichlid *Pseudotropheus* (*Maylandia*) 'zebra gold', species polymorphic for P/OB colouration. Moreover, I find evidence for genetic inheritance but no evidence of imprinting on sibs' colouration as a causal origin of individual male mating preferences.

CHAPTER V examines the possibility that blotched polymorphisms have the potential to generate new species as the result of fixation of alternative colour and sex determining factors in different populations and sexual selection by male choice. An ideal study species is identified, *Paralabidochromis chilotes*, a Lake Victoria rock-dwelling species, fixed for P morph in most of its range but female polymorphic at one island site and fixed for WB female morph at an adjacent island site. I show that male mating preferences have coevolved with female colour patterns and that strong male mating preferences between parapatric island populations are sufficient to determine full reproductive isolation, as confirmed by assortative mating experiments in the lab as well as a population structure analysis. The latter reveals patterns of restricted gene flow only between the population fixed for blotch and all other non-blotched populations that represent a large P metapopulation with gene flow regulated only by geographical distance. In the absence of strong ecological divergence, the population fixed for blotch is reproductively isolated by male mating preferences and might represent the first documented case of speciation with gene flow driven by sexual selection by male mating preferences on female colour polymorphism.

In CHAPTER 6, I consider some unresolved issues and possible future lines of research emerging from this thesis.

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CHAPTER I

SEX DETERMINATION, SEX LINKAGE AND THE EVOLUTION OF MALE MATING PREFERENCES FOR SEX-LINKED COLOUR PATTERNS

Part I

The evolution of sex determining systems and sex chromosomes: a story of conflict, demise and re-birth.

The mechanisms underlying sex determination are extremely diverse (Bull, 1983). Sex can be determined by the action of genes with little environmental influence (genetic sex determination, GSD) or by temperature, pH, sex ratio, density, social dominance (environmental sex determination, ESD), although this distinction is over simplistic and probably largely artificial, with pure GSD and ESD connected by a continuum of sex determination systems (Shine et al. 2002; Sarre et al. 2004) with complex pleiotropic and epistatic effects. Genetic sex determination can take the form of a 2-factor system with either male or female heterogamety, or three or more sex factors can segregate in the same population (multiple factor systems, *sensu* Bull 1983). Polyfactorial systems, in which sex determination is set by many genes with small additive effects, are not uncommon, too (Peters, 1964; Yusa & Suzuki, 2003). Sex can also be determined by heterozygosity at a single locus (complementary sex determination) (Whiting, 1939; Cook, 1993), or by the ratio of sex chromosomes and autosomes (Penalva & Sanchez, 2003). Finally, sex determination does not need to be directed by nuclear genes but cytoplasmic factors either in the form of mitochondrial genes or cytoplasmic parasites, such as the endosymbiotic bacteria of the genus *Wolbachia* can act as sex determining factors (Werren & Beukeboom, 1998; Zeh & Zeh, 2005).

Although alternative pathways are possible, it is likely that the origin of sex determination coincided with the establishment of dioecy from a cosexual state, as common in most flowering plants (Charlesworth, 2000). This required a male sterility mutation converting cosexuals into females, and a female sterility mutation, converting cosexuals into males. Selection might have favoured this transition possibly because sexual individuals would have reduced the production of unfit offspring, as a result of self-fertilisation, and because reallocation of reproductive resources into one sex, only, might have led to an increase in fertility (Charlesworth, 1996). Recombination would have generated sterile individuals carrying both mutations, so the invasion of the second mutation required some level of linkage to the first. Selection for reduced recombination between the two mutations eventually led to the rise of primitive sex chromosomes, in which the two sex-specific sterility genes were inherited at a single locus. This scenario explains the widespread observation that in young sex determining systems (such as young plant taxa that recently evolved dioecy from hermaphroditism or newly established sex determiners) differentiation between sex chromosomes is limited to a small region of restricted recombination around the sex locus (Westergaard 1958, Liu et al. 2004).

Once a chromosome has evolved a sex determining factor, it will be exposed to the accumulation of sexually antagonistic genes, i.e. genes beneficial to one sex but detrimental to the other, in linkage with the sex determining factor. Suppression of recombination between sex locus and sexually antagonistic loci will be favoured, initiating the process of degeneration of the sex chromosome. Directional selection acting on one locus will interfere with selection at other loci that happen to be in the non-recombining region (hence, in physical linkage): as a consequence, the effects of both background selection and genetic hitchhiking will be large, leading to the fixation of mildly deleterious mutations on the emerging sex chromosome (Charlesworth & Charlesworth, 2000). Moreover, in the absence of recombination and back mutation, the stochastic loss of the class of sex chromosomes with the least mutations will progressively lead to the fixation of both mildly and strongly deleterious mutations by Muller's ratchet (Muller, 1964; Charlesworth, 1978).

The insertion of transposable elements in this decaying region will have progressively smaller deleterious fitness consequences and will not be counteracted by the purging action of recombination. Transposable elements will also generate similar sequences at different sites on the sex chromosome, favouring chromosomal rearrangements which might enlarge the region of no recombination.

The accumulation of sexually antagonistic genes and transposable elements, background selection, genetic hitchhiking, and Muller's ratchet will interact in a positive feedback, accelerating the degeneration of the emerging sex chromosome.

The progressive degeneration of genes on the sex chromosome also creates the need for dosage compensation, a process achieved with very different mechanisms in different taxa (Baker et al. 1994; Nguyen & Disteche 2006). If dosage compensation does not act on a gene-by-gene basis but by regulating entire blocks of genes at a time, the degeneration of the sex chromosome might become an adaptive process, when functionally intact genes on the sex chromosome become dosage compensated on the other chromosome pair (Orr & Kim, 1998).

Progressively, the sex chromosome is expected to lose most of its functional genes with the exception of genes with a role in sex-specific processes or potentially sexually antagonistic (e.g. in the Y chromosome, genes with a role in spermatogenesis, genes for costly ornaments under sexual selection, etc.).

Indeed, it has been suggested that the Y chromosome should experience a process of masculinisation while the X chromosome might become masculinised or feminised, as a consequence of the accumulation of sexually antagonistic genes on the sex chromosomes.

Y's guys and Xtreme sex chromosomes: masculinisation and feminisation of the genome

Fisher was the first to suggest that sex-linked mutations beneficial to one sex and deleterious to the other would be shielded from selection when rare (Fisher, 1931). Rice (1984) subsequently developed Fisher's ideas modelling the conditions for invasion and the equilibrium frequencies of mutations with different levels of dominance rising on autosomes or sex chromosomes. He observed that a largely recessive mutation arising on the X (on the Z) chromosome beneficial to males (to females) but deleterious to females (to males) would be able to increase in frequency as long as it is in heterozygous individuals, even if this mutation is lethal when homozygous in the homogametic sex. Also, since X chromosomes spend 2/3 of their time in females (and Z chromosomes 2/3 of their time in males), dominant mutations favouring the homogametic sex are exposed to selection more in the sex where they are beneficial. As a consequence, a sufficiently dominant mutation benefiting the homogametic sex will increase when rare even when the cost to the heterogametic sex far exceeds the benefit to the homogametic sex. Finally, mutations beneficial to one sex but deleterious to the other (i.e. sexually antagonistic) are also expected to accumulate in linkage with the sex determining locus, in this way achieving sex-specific expression and hence resolving sexual antagonistic conflict (intra-locus ontogenetic conflict of Rice & Chippindale, 2001).

We therefore expect to find a prevalence of genes beneficial to males and deleterious to females on the Y chromosome and genes beneficial to females and deleterious to males in W chromosomes. Moreover, recessive female advantageous mutations should accumulate on Z and dominant female advantageous on X. Recessive male advantageous mutations should be found more frequently on X and dominant ones on Z. This set of predictions implies a new concept, the idea that genes are not randomly distributed throughout the genome.

When Rice's (1984) analysis was published, very little information was available on the genetic content of the sex chromosomes even for intensely studied model systems such as *Drosophila*, mouse and human. However, in the last 6 years, a burst of studies has dramatically changed the situation and evidence is rapidly accumulating in favour of a biased distribution of genes with antagonistic properties on the sex chromosomes.

There is evidence for an enrichment of genes with female-biased expression on the X chromosome of the mouse, *Drosophila melanogaster* and *Caenorhabditis elegans* (Khil et al. 2004; Ranz et al. 2003; Reinke et al. 2004). The X chromosome is also expected to accumulate genes with male-biased expression but early studies in mammals fail to detect such effect. The reason might have to do with meiotic sex chromosome inactivation (MSCI), a process by which, during male meiosis, the sex chromosomes are transcriptionally muted. Beneficial male genes required during this phase might become entrapped on sex chromosomes and selection would then favour an autosomal localisation. However, if beneficial male genes are expressed before MSCI, we would expect accumulation on the X chromosome in line with Rice's models. Indeed, a study on the mouse showed that genes expressed before the onset of MSCI were over-represented on the X but genes expressed after MSCI were under-represented on the X (Khil et al. 2004). As attractive as this hypothesis can be, it does not explain a similar lack of over-representation of male beneficial genes on the X in *Drosophila* and *C. elegans*, where MSCI is absent (Reinke et al. 2000; Jiang et al. 2001; Kelly et al. 2002; Meiklejohn et al 2003; Parisi et al. 2003; Ranz et al 2003). Interestingly, a recent study analyzing transcription profiles obtained by EST data from different tissues in the chicken (a species with ZW/ZZ system) revealed over-representation of male-biased genes and under-representation of female biased genes on the Z chromosome (Kaiser & Ellegren, 2006). This result as well as similar findings in *Drosophila* and *C. elegans* might not conflict with Rice's (1984) predictions as long as mutations were found to be at least partly dominant. Evidence that sexually antagonistic alleles are dominant gain-of-function mutations, however, is still scarce (Ranz et al. 2003).

If a gene is potentially beneficial to males but detrimental to females, Y-linkage in a male heterogametic species would restrict its expression to the sex in which it is beneficial; with a similar argument, W-linkage in a ZW/ZZ system might be expected for genes potentially detrimental to males but beneficial to females.

The mammalian Y chromosome, once considered a "genetic wasteland", is in fact known today to contain 76 protein-coding genes, and at least 27 proteins have been identified. A large fraction of them is specialised in spermatogenesis (Lahn & Page 1997; Skaletsky et al. 2003). A biased distribution of genes on the sex chromosomes is achieved by the re-functionalisation or functional specialisation of genes already in situ and by active retrotransposition on and off the sex chromosomes (Emerson et al. 2004; Khil et al. 2005). Of all transposed sequences, most will rapidly degenerate to pseudogenes (Carvalho & Clark, 2005) but a few will be able to acquire a sex-specific function and be positively selected.

In 2003, a team led by David Page (Rozen et al. 2003) made the exciting discovery that the male-specialised Y chromosome in humans is maintained against degeneration by gene conversion, via recombination between Y sequences. This is made possible by the presence of palindromic sequences flanking the chromosomal region with male-specific function. The frequent insertion of repetitive elements on the sex chromosomes, as part of their degeneration process, might create the opportunity for the evolution of such palindromes and in a twist to the story, contribute to counteract sex chromosome degeneration.

I will go back to the issue of a biased distribution of genes with antagonistic effects on the sex chromosomes in the following paragraphs, as this might have profound consequences both on the operation of sexual selection and, as I will argue, possibly on the maintenance of a certain sex determining system and the likelihood of a reversal in heterogamety.

The accumulation of these genes in tight linkage with the sex determining locus will only retard the degeneration of the whole sex chromosome and its eventual loss, as in mole voles (Just et al. 1995). Before a Y(W) chromosome is lost, those genes with male (female) biased function, that were maintained or even actively imported, will be duplicated or translocated to other chromosomes. Given the limited size of the sex-specific region containing the master sex determiner and the set of genes with sex-specific functions, it is possible that transpositions in this region might export a large proportion of such genes to a single autosome, making it a good candidate for the role of new sex chromosome. In this way, a master sex determiner might survive the sex chromosome in which it is embedded by fleeing in time from the sinking carcass of the old sex chromosome. Alternatively, a new sex determiner on a different chromosome will take over the role of master sex factor. Either way, this will mark the beginning of a new cycle of sex chromosome degeneration.

This leads us to the issue of how and why new sex determiners arise in a population.

The rise of a new master

The loss of a sex chromosome following the complete degeneration of its gene content is only one situation in which a new sex determiner might arise. Rather, early stages in sex chromosome evolution following the establishment of a new sex determiner might also be the most favourable for the take-over of the system by another novel sex determiner. When a new sex chromosome has arisen, transposable elements and repetitive sequences quickly start accumulating, as described above. Both the accumulation of repetitive elements and the frequent occurrence of duplications and translocations will favour rearrangements via non-homologous recombination of chromosomal segments (McDonald 1998). This increase in the local frequency of rearrangements might have a direct active role in the generation of new sex determiners. Indeed, it has been suggested that the variability of sex determining systems in poeciliid fishes is due to frequent deletions and duplications in the sex determining region, affecting the expression of dose-sensitive sex determining factors (Volff & Schartl, 2001). A series of pericentric inversions, and possibly duplications, on the X chromosome led to the evolution of XX/XY and ZW/ZZ heteromorphic sex chromosomes in different populations of the frog *Rana rugosa*, from an ancestral homomorphic XX/XY system (Miura et al. 1997; Ohtani et al. 2000). As a consequence of duplications in the human gene DAX-1 in the dosage-sensitive sex reversal region of the X (DSS), XY individuals develop as females. DAX-1 is an inhibitor of testis development and its increased expression is probably sufficient to override the effects of the master male determiner Sry (Swain et al. 1998).

More examples of such processes potentially leading to the emergence of new sex determiners are now available and many involve teleosts. I will describe them in the context of the evolution of sex determining system in fishes and show that such rearrangements represent a common theme of sex chromosome evolution in this group.

Independently from the molecular mechanisms behind the rise of a new sex determiner, a general observation can be made: master sex determiners are not phylogenetically conserved sometimes even across closely related species. Mank et al. (2006) found extreme evolutionary lability of the sex determination system in teleosts, with multiple frequent transitions between alternative heterogametic systems as well as between gonochorism and hermaphroditism, within families and sometimes even between species or populations of a single species. Evidence for frequent transitions between male and female heterogamety is also abundant in other vertebrates (amphibians: Hillis & Green, 1990; reptiles: Kraak & Pen, 2002).

The flexibility of the system of sex determination is in stark contrast with other developmental pathways, which appear to be largely highly conserved across even phylogenetically very distant taxa (Wilkins, 2002). This might have to do simply with the fact that a sex determining system essentially is only required to code for a 0/1 signal (male or female) and upstream disruptions along the regulatory chain do not determine loss of essential metabolic functions but a switch from one sex to the other. Such extreme upstream diversity does not apply to downstream regulators in the pathway: these in fact show surprising conservation or at least a long time involvement in sex regulation across distant taxa, suggesting that strong selection is involved in their conservation. In particular, pleiotropy might play an important role as it is likely that multiple genes are the targets of regulators at the bottom of the sex determination pathway, as opposed to upstream ones responding to and affecting respectively the gene that precedes them and the gene that follows them in the linear pathway. Therefore, the potentially complex network of targets of bottom regulators might prevent frequent downstream changes.

Wilkins (1995) proposed that a sex determination pathway evolves backwards by addition of upstream regulators, recruited from pre-existing genes with functional redundancy. This process of upstream addition is driven by frequency-dependent selection on the sex ratio. This hypothesis predicts downstream conservation of genes and functions, as downstream factors are supposedly the oldest in the pathway, while factors at the top should be highly variable as they represent the most recent additions. Wilkins' model nicely explains the puzzling variety of sex determining systems that characterise even closely related species and sometimes populations of the same species, and at the same time, the striking observation that some genes have a long history of involvement in sex regulation. For example, dsx has been long involved in sexual development in insects (Saccone et al. 2002), and a similar DM-domain gene, mab-3 is a downstream regulator in *C. elegans* (Shen & Hodgkin, 1988; Raymond et al. 1998). Dmrt1 is conserved in flies, worms and mammals (Raymond et al. 2000), Sox9 and WT1 are conserved from fish to mammals (Kent et al. 1996), AMH from reptiles to mammals (Sinclair et al. 2002).

The continuous addition of upstream regulators would generate a cascade of ever increasing length, which is unlikely to be stable. Schartl (2004) has suggested that duplication of a gene D at an intermediate position in the cascade A → B → C → D → E → F → creates a copy D' of the gene with basal level of gene expression. The levels of gene product generated by the two copies (D, D') might be sufficient to trigger the activation of the downstream factor E without the need for the gene C upstream the duplicated gene D to trigger its activation. In this way, the whole upstream chain of regulators (A → B → C →) becomes dispensable and decays. The duplicated gene will be able to rise to the role of new master sex determiner, when mutations on regulatory regions of the duplicated gene confer constitutive expression, making one of the two copies dispensable. Eventually, under sex ratio selection, a new upstream regulator X might be added, re-starting the process of bottom-up evolution from the duplicated gene D' (i.e. X → D' → E → F →).

This scenario is particularly suitable to explain how a gene involved in sex development can become the master switch (the opposite of Wilkins's suggested direction of evolution) as it appears to have happened in the medaka's sex determining gene Dmrt1bY (discussed below).

The invasion of a new sex determiner might be driven by selection, acting either 1. directly on the sex determining factors, or 2. on sex-linked traits.

1. The sex determining factors might be under direct selection in different scenarios: a population might face ecological changes which might affect competition, reproductive strategies etc., favouring changes in the local optimal population sex ratio. Selection on new determiners in a two-factor system or selection on a specific sex factor in multiple-factor sex determining systems (*sensu* Bull, 1983) would drive these shifts in the sex ratio towards the new optimal population sex ratio.

Sex ratio selection is also at the heart of the evolution of sex determining systems by genetic conflict (Werren & Beukeboom, 1998). Conflicts between cytoplasmatically inherited elements and Mendelian nuclear genes might generate reversals of the sex determining systems and sometimes even cycles in which new nuclear sex determiners alternate between periods of cytoplasmic determination. In the isopod *Armadillium vulgare*, a female heterogametic system with homomorphic sex chromosomes is normally in place. However, many populations are infected by the maternally inherited cytoplasmic bacterium *Wolbachia*. Among the various effects on its host, *Wolbachia* induces sex reversal of genetic males into functional females. The cytoplasmic sex ratio distorter can spread in the population and lead to the loss of the sex chromosome (W), with all individuals being ZZ and sex being determined by infection. Sex ratio selection resulting from the spread of *Wolbachia* favours the evolution of masculinizing factors. Indeed, some populations harbour an autosomal dominant masculinizing gene M that overrides the feminising effect of *Wolbachia*. The increase in frequency of M, driven by sex ratio selection, leads to a new sex determining system in which infected individuals are males when carriers of the M allele and females otherwise (mm+Wo/Mm+Wo). In other words, M becomes the new sex determiner and a male heterogametic system is established. Invasion of a new cytoplasmic sex ratio distorter epistatic on M will restart the cycle and possibly lead eventually to a female heterogametic system.

Inspired by the *Armadillium* system, Caubet et al. (2000) modelled the invasion of a cytoplasmic sex ratio distorter and showed that the genetic conflict between cytoplasmic and nuclear factors can drive the evolution of a new sex determining system. After the invasion of the cytoplasmic element, the genetic sex determiner is rapidly lost and the invasion of a dominant masculinizing repressor of the cytoplasmic element is favoured. Its appearance and increase in frequency leads eventually to a system of genetic sex determination with opposite heterogamety, but this only as long as the repressor has no cost and represses the sex effects and not the transmission of the cytoplasmic element. This also illustrates an important point, possibly sometimes neglected in the search for putative sex determining genes. The master sex determiner is not necessarily involved in the sex determination cascade: if we imagine the coevolution of cytoplasmic and nuclear factors (Bull, 1983) the invasion of a population with female (male) heterogamety by a cytoplasmic sex ratio distorter might favour the evolution of resistance genes. The system can eventually evolve, through loss of the original sex determiner, to a condition where the population is all infected and has reversed to male (female) heterogamety at the resistance locus, which represents the new master sex determiner. However, this factor is not involved, at least not in a conventional way, into the sex determination cascade, but might just be a growth inhibitor of the cytoplasmic element. This suggests that the search for the master sex determiner might be for some groups particularly challenging since multiple sex ratio distorters, nuclear as well as cytoplasmic, can be harboured in natural populations undetected, and because the master gene function (and so, its structure) need not be related to the regulation of downstream factors in the sex determining pathway.

Nuclear genes such as parental-effect sex determiners (PSD), expressed in the parent but acting in the zygote, have the potential to create conflict over the sex ratio and shape sex determining systems. Maternal-effect sex determiners are often maternal mRNAs or proteins placed in the egg and expressed by nuclear genes of the mother as in the case of *daughterless* (*da*) in *Drosophila* or similar nuclear genes in *Musca.* Cases of paternal-effect genes are also known (e.g. *psr* in *Nasonia vitripennis*). The interest in such systems is due to the observation that in the context of partial inbreeding or subdivided populations with local mating in temporary demes, the optimal sex ratios for the mother and for the offspring diverge. In particular, Werren and Hatcher (2000) found that mothers favour a more female-biased population sex ratio than their offspring. However, models (Kozielska et al. 2006) considering polymorphisms in zygotic sex determiners but excluding maternal control, do not reach similar results to Werren et al. (2002) and it is unclear how common maternal-effect sex determiners are. Moreover, the prediction that mothers would favour a more female-biased population sex ratio is not universal. As pointed out by Pen (2006), the result depends on parameters such as dispersal and mating system: for example, with female dispersal and polygyny, offspring favour a more female biased population sex ratio than their mothers, the reason depending on differences in relatedness between parent and offspring and between offspring affected by local mate competition.

Last, if new sex factors have some pleiotropic fitness advantage, positive selection might increase their frequency, and under certain circumstances, the new sex determiners might take over sex determination. The recent observation that genes relatively upstream in the sex determination cascade are directly involved in other pathways regulating mating behaviour, courtship and aggression (Wolfner, 2003; Skuse, 2006; Vrontou et al. 2006) suggests that the dynamics of sex determination might directly influence and be heavily influenced by shifts in life history, exploitation of new niches, mating strategies, etc. This in turn might help explain the apparent flexibility and diversity of this developmental pathway. Let's imagine for example, for a certain species, that a condition for the exploitation of a new niche is an increase in aggressive behaviour leading to the evolution of territoriality. Duplication of genes of the sex determination pathway and pleiotropically involved in the hormonal control of aggressiveness would be favoured since it would provide increased expression of factors stimulating aggression. However such duplication might have dramatic effects on the sex determination cascade, too. The duplicated copy, in fact, might escape the close range cis-regulation and a process of restructuring of the sex determination cascade, as envisaged by Schartl (2004) and described above, might be started.

It is important to notice that when a sex factor is under positive selection, sexually selected traits tightly linked to it might hitchhike and increase in frequency in the population, this depending on the net fitness effects deriving from natural and sexual selection on the sexually selected trait (which might even be frequency-dependent) and the fitness advantage associated with selection on the sex factor.

2. Selection on a trait tightly linked to the sex determining factor. If changes in the environment, predators, parasite community etc. modify the fitness value of the sex-linked trait (e.g. conspicuousness of a sexually selected trait in a predator-free environment that is invaded by new visual predators), selection on the trait will also affect, by hitchhiking, the sex factor linked to it. As a consequence a new sex determining system can establish by hitchhiking on a favourable trait. Therefore, different populations characterized by differences in the fitness of the sex-linked trait might develop different sex determining systems. The house fly *Musca domestica* has a XY sex determining system in most of its range. However, some populations of this species were invaded by a new male determiner M which is believed to be associated with DDT resistance, although it is not clear whether this is a pleiotropic effect of M or a distinct gene

physically linked to M conferring resistance (Franco et al., 1982). Interestingly, a new feminizing factor F has evolved and is present only in the populations invaded by M, probably as a result of sex ratio selection generated by the masculinizing effects of M. Divergence in sex determining systems between populations might lead to hybrid incompatibilities and hence to partial or complete postzygotic isolation. Another possibility that I will suggest in a following paragraph is that a change in sex determining system might affect the genomic distribution of sexually antagonistic genes, increasing the likelihood for the origin of hybrid incompatibilities, as a consequence of i. the redistribution of such genes to different chromosomes and ii. their frequent role in reproduction.

The accumulation of sexually antagonistic genes and repetitive sequences in proximity to the sex locus in the early stages of sex chromosome evolution attracts also unwanted guests: driving loci. These ultraselfish genetic elements can invade a population thanks to the suppression of recombination in this region of the genome. In fact, a so-called 'ultraselfish killer complex' requires little or no recombination between two interacting loci, the drive locus and a responder locus on the homologous chromosome, in order to avoid the formation, by recombination, of a suicidal chromosome. Sex chromosomes, typically characterised by reduced recombination, therefore provide an ideal location for such complexes. Nuclear ultraselfish elements, such as meiotic drive complexes, can have effects on the sex ratio similar, to some extent, to the cytoplasmic ultraselfish elements mentioned above, when they invade in linkage with sex determiners. In sex chromosome drive, X or Y (Z or W) are preferentially transmitted into the next generation. X-chromosome drive has been detected in fruitflies, mosquitoes and lemmings. In the context of this thesis, the condition found in lemming species is of particular interest, as their sex chromosome system bears similarities with the sex determining system of the cichlid fish *Neochromis omnicaeruleus* and other haplochromine cichlid species studied in the present work. In the varying lemming *Dicrostonyx torquatus*, dominant female determiners are present in the form of X chromosomes (X*) suppressing the male function of Y chromosomes and resulting in phenotypically functional females X*Y. Such individuals regularly produce X* and Y eggs so that half of their Y gametes generate YY sons that are inviable. As a consequence, 2/3 of an X*Y female's offspring (and not ½) will be X* carriers. This transmission advantage is apparently not counteracted by the reduced fecundity associated with the production of inviable YY sons: in fact, rodents are known to produce more eggs than they actually implant (and the number of embryos being implanted is lower than the number that are carried to birth) so varying lemmings can compensate for the loss of YY individuals by overproducing gametes. Hence even when all female genotypes (XX, XX*, X*Y) have equal fecundity, X* will increase in frequency until sex ratio selection will counterbalance its transmission advantage and an equilibrium will be reached (Bull & Bulmer, 1981). Observed frequencies in the wild however hint at a more complex picture (Gileva et al. 1982) and there is some evidence that XY males might overproduce sons, suggesting the intervention of Y drive, too, in the system. This might be expected in the conflict between feminising X* and autosomal genes overproducing Y-bearing sperm. However, the conflict is in reality penalising X and not X*, since males are X carriers. Hence, it is the X chromosome that will decrease in the population as a consequence of overproducing Y-sperm (Y drive) and not X*, which will increase at the expense of X, biasing the population sex ratio even more towards females.

X*Y females are present also in the wood lemming *Myopys schisticolor* but in this species X* drives, i.e. females produce only X* eggs (Winking et al. 1981), by double non-disjunction in the female germline resulting in YY cells that lack essential genes and therefore are precociously lost. X* in this species has therefore a 100% transmission and as a result the population sex ratio will be heavily female biased. In this species X* appears to have some intrinsic fitness advantage since X*Y females are heavier at birth, mature faster and have a higher pregnancy rate than X*X females and these in turn are superior to XX.

In another rodent, the vole mice *Akodon* spp., XY females are present but in this case a mutant Y (Y*) is responsible for sex reversal (Bianchi & Contreras, 1967; Hoekstra & Edwards, 2000).

Interestingly, also in this case there is evidence that XY* females are superior to XX females, reproducing earlier, having a longer reproductive life and a shorter interval between litters, and an estimated fitness 35% higher than XX (Hoekstra & Hoekstra, 2001; Burt & Trivers, 2006). In this system, compensation does not favour the mutant sex chromosome, given the inevitable loss of YY* individuals. It has been suggested that the XY reversal in *Akodon* rodents might be the result of conflict between mitochondrial genes and Y genes (Zeh & Zeh, 2005). The higher growth rates before implantation in XY* embryos might be the result of selfish growth factors on the Y* evolved as a mitochondrial response to selfish Y growth factors identified on the mammal Y chromosome and known to induce faster pre-implant growth of male embryos. As a result of the accumulation of selfish growth factors on the Y*, XY* females produce 2/3 female offspring, passing on the mitochondrial haplotype to the next generation. Moreover, although YY* inviable embryos are formed, overproduction of oocytes allows for reproductive compensation. Hoekstra (2003) showed that mitochondrial haplotypes associated with XY* mothers have a transmission advantage of 1.33 and can potentially rapidly sweep to fixation. These examples from rodent species are interesting because they provide valuable comparisons with the platyfish 3-factor system and the cichlid *Neochromis omnicaeruleus* system in which a dominant female determiner on the X chromosome (what we would indicate as an X* chromosome, in "lemming notation") has invaded a population with XX/XY system. I will come back to this point and the potential for the *Neochromis* X* to be a driving chromosome in a following paragraph.

I have so far focused on the effects of selection for the invasion of sex determining genes. However, a new sex factor can invade and increase in frequency even in the absence of either a pleiotropic fitness advantage or tight linkage with a gene under positive selection or when sex ratio selection is generated by the presence of ultraselfish elements.

Vuilleumier et al. (2007) recently showed that sex ratio selection and genetic drift alone can sustain the invasion of a dominant sex determiner and drive a complete reversal of heterogamety in a population. The authors modelled the invasion of a dominant female determiner W with no individual fitness advantage, in a male heterogametic system. Vuilleumier et al. (2007) considered a metapopulation consisting of a finite number of demes of similar size, with dispersal across demes occurring at random. Genetic drift was included to account for the finite population sizes. The probability of fixation of a mutant W was investigated at different population sizes and, in a second analysis, the effects of metapopulation structuring and connectivity on the probability of fixation of W were also studied. Sex chromosomes were considered to be at an early stage of differentiation, i.e. with free recombination outside the sex-specific region of the Y and mildly deleterious mutations beginning to accumulate: hence models with or without a fitness disadvantage to YY males were considered.

Vuilleumier et al (2007) were able to show that the interaction between genetic drift and sex ratio selection is sufficient, even in the absence of metapopulation effects and even when YY have moderate disadvantage, to support the invasion and fixation of W, in all cases the fixation probability being higher than the corresponding one for a neutral gene with no involvement in sex determination. Interdemic selection generated by metapopulation structuring increased the probability of fixation for intermediate levels of subdivision. Although the time to fixation was negatively affected by increasing migration rates, yet relatively high connectivity optimised the probability of W fixation. In conclusion, and complementing Bull's (1983) original analysis on the 3-factor sex determining system of the platyfish (see also below), Vuilleumier et al. (2007) showed that a dominant female determiner W can invade without any individual fitness advantage and reach fixation in a finite population simply through the interaction between sex ratio selection and genetic drift, even when YY individuals have slightly reduced viability. Their analysis also suggest that fixation of W is favoured by structuring with relatively high dispersal between demes, as is likely to take place in the complex metapopulations of haplochromine cichlids species of Lake Victoria and Malawi.

Sex determination and sex chromosome evolution in teleost fishes

The available information on the sex determining system and the nature of sex chromosomes in different fish families is largely anecdotic, with few notable exceptions.

Extensive cytogenetic research involving species of neotropical freshwater fishes and particularly of the order Characiformes (de Almeida Toledo & Foresti, 2001) has revealed an astonishing variety of sex determining systems in closely related species and often in different populations of the same species, this variation originating by frequent chromosomal rearrangements. Both conservation of heterogametic sex within families and coexistence in the same family of male and female heterogamety as well as multiple sex chromosome systems were reported (Moreira-Filho et al. 1993; de Almeida Toledo & Foresti, 2001). For example, in the widely distributed characiform *Erythrinus erythrinus*, Bertollo et al (2004) were able to reconstruct a possible pathway connecting four distinct chromosomal types generated by successive centric fusions and different pericentric inversions. These resulted in three chromosomal races having different multiple sex chromosomes of the type $X_1X_1X_2X_2/X_1X_2Y$, and one race with undifferentiated sex chromosomes.

Multiple sex chromosomes have been detected in other species of neotropical freshwater fishes. A $X_1X_1X_2X_2/X_1X_2Y$ system has been reported in the family Cyprinodontidae (Uyeno & Miller, 1971) and in Goodeidae (Uyeno & Miller, 1972), as a result of Robertsonian fusions. Interestingly, in several characiform species (De Almeida Toledo & Foresti, 2001) and in two gymnotiform species (De Almeida-Toledo et al. 2000), this multiple chromosome system originated via more complex rearrangements probably involving translocations and pericentric inversions. A different condition is present in the characiform *Hoplias malabaricus* males have one chromosome more than females as a consequence of a fusion/fission process, leading to a XX/XY_1Y_2 system.

In *Apareiodon affinis* (Characiformes, Parodontidae), a ZZ/ZW_1W_2 system resulted from a centric fission involving the original W, leaving males with one chromosome less than females. But in the lizardfish *Trachinocephalus myops* (Siluriformes, Synodontidae) a similar ZZ/ZW_1W_2 system was achieved with a completely different mechanism, i.e. by centric fusion of the ancestral Z chromosome with an autosome, generating a neo-Z. The autosome, whose pair was fused with the ancestral Z, became a W (W_2), while the ancestral W maintained its integrity (W_1). This last comparison suggests that the specific processes leading to the establishment of a sex chromosome system can be very different: these differences will largely determine the fate of genes linked to the ancestral sex chromosomes and, in particular, whether the new sex determining system will initially generate sexually antagonistic conflict.

Together with these examples of extreme genomic plasticity and variation in sex determining systems, teleost fishes provide good examples of well differentiated and highly conserved sex chromosomes: Ota and his collaborators (Ota et al. 2000, 2003; Ueno et al. 2001) found that a ZW/ZZ system with degenerated W chromosomes is shared among species of the family Synodontidae and by the closely related family Aulopidae (from which Synodontidae separated more than 60 million years ago). The authors suggest that the same sex chromosomes have been conserved in the two families and conclude that the age of the Synodontidae-Aulopidae ZW/ZZ sex chromosomes might be comparable with the age of the avian ZW/ZZ (60-100 my).

The Aulopiformes represent an extremely interesting group to study the evolution of sex determination for another reason: this order comprises both gonochoristic and synchronous hermaphroditic species recently derived from gonochoristic ancestors. Ota et al. (2000) found heteromorphic sex chromosomes in the gonochoristic species but no evidence for heteromorphism in the hermaphroditic species, suggesting that heteromorphic sex chromosomes were secondarily lost. Molecular phylogenetic and cytogenetic analyses confirmed the loss of the sex chromosomes with consequent reduction of chromosome number, together with large scale genomic rearrangements. The loss of the sex chromosomes in hermaphroditic species, in the context of the well differentiated and stable sex determining system of the Aulopiformes, raises new questions on the interplay between sex chromosome evolution and life history, particularly mating strategies. It is possible that in the final stages of degeneration of the Aulopiformes' sex chromosome, its eventual loss in some species was not replaced by a new master sex determiner arising on a different chromosome or by a translocation of the ancestral sex factor from the degenerated W to an autosome. Instead, at that stage, selection favouring a shift to synchronous hermaphroditism, i.e. favouring the expression of both sexes in the individual, driven by ecological selection, might have opposed the rise of a new master switch in the sex determination pathway.

It is surprising that no information is available yet on the mode of sex determination in the three fish species for which a genomic map is available, the zebrafish *Danio rerio* and the two pufferfish species *Tetraodon nigroviridis* and *Takifugu rubripes* (Li et al. 2002). But this might not be so for long: candidate genes have been recently proposed for the zebrafish (von Holsten & Olsson, 2005) and sex-linked markers have been identified in *T. rubripes* (Cui et al. 2006). However, while the sex determining region of a handful of teleost species has been identified so far, only in one case the sex determining locus has been successfully isolated.

Salmonids have been long the object of intense research for their commercial value and what we know today about the evolution of their sex determining system gives us another glimpse at the various processes that can directly affect the degeneration of a sex chromosome. The family Salmonidae is known to have a XX/XY sex determining system (Phillips & Rab, 2001) but chromosome painting data suggested that different Y chromosomes have independently evolved multiple times (Phillips et al. 2001).

A comparison of the Y chromosome linkage maps of four species representative of the three major genera of the salmoninae (rainbow trout, *Onchorhynchus mykiss*; lake trout, *Salvelinus namaycush*; Arctic charr, *Salvelinus alpinus*; Atlantic salmon, *Salmo salar*) confirmed that sex chromosomes are not conserved between species (Woram et al. 2003). Notably, arrangements of markers close to the sex determining locus were conserved on homologous (but autosomal) linkage groups, suggesting that the sex determining region has moved to a different chromosome multiple times in the history of the salmonid radiation. In support of this hypothesis, the sex determining region is generally found (with the exclusion of the rainbow trout) at the end of the Y linkage group and there is recent direct evidence that the sex locus is located in telomeric position on the short arm of the largest chromosome in both coho and chinook salmon, although the sex linkage groups are not conserved (Phillips et al. 2005). If the sex determining region has a telomeric position, it can be more easily translocated to a new chromosome. We can then imagine multiple translocations (or possibly, transpositions) of short chromosomal segments containing the sex determining factor during the salmonid radiation, generating new sex chromosomes but maintaining both the master sex determining genes and the heterogametic system. If this scenario is correct, it would not be surprising that heteromorphic sex chromosomes are rare in salmonids (Hartley 1987), despite the conservation of a XX/XY system for a long period of time. But there is a twist to the story. Dosage compensation following the initial restriction of recombination between sex chromosomes is thought to be an important factor contributing to the degeneration of Y (or W). Salmonids have experienced a tetraploidization event early in their evolutionary history (Allendorf & Thorgaard, 1984) and this might have dispensed the genome from the need of dosage compensation. In turn, the degeneration of the sex chromosome in the absence of dosage compensation might, as a consequence, be less pronounced in salmonids, explaining the largely homomorphic sex chromosomes of this group.

In sticklebacks, both male and female heterogamety have been found and some species have homomorphic sex chromosomes while others exhibit cytologically well differentiated sex chromosomes (Chen & Reisman, 1970). Peichel et al (2004) have recently identified the master sex determining locus of the threespine stickleback (*Gasterosteus aculeatus*) at the distal end of linkage group LG19, in a region with unusually low recombination rate and marked by the accumulation of transposable elements and duplications, as characteristic of early sex chromosome evolution. Probing sex-linked markers from *G. aculeatus* in the closely related species *G. wheatlandi*, thought to have diverged from the threespine stickleback around 10 million years ago, showed no sex specific location in *G. wheatlandi* for any of the genes sex linked in *G. aculeatus*. Since *G. wheatlandi* has an XX/XY system with heteromorphic sex chromosomes, the authors suggested that the sex determiner of *G. aculeatus* probably arose after the divergence of these two species. Peichel et al.'s (2004) findings are of particular interest because they allow investigation in the early stages of sex chromosome evolution and, in the future, comparative analyses between related species at different stages of sex chromosome degeneration as well as between related species with alternative heterogamety. As the specific master sex determiner has not yet been identified, it is premature to propose a possible evolutionary pathway that led to the establishment of the threespine stickleback sex determiner. But it is tempting to speculate around the terminal position of the sex determining region in the linkage group and the possibility that this region was translocated from a different chromosome, eventually assuming the role of master sex determiner in an instantly created area of non-recombination.

In 2002, a major breakthrough in studies of sex determination was achieved: the first sex determining gene in a non-mammalian vertebrate was isolated by Matsuda and his collaborators (Matsuda et al. 2002) in the medaka *Oryzias latipes*, a species with XX/XY sex chromosome system. The gene DMY (or dmrt1bY) encodes a putative transcription factor containing a zinc-finger DNA binding domain. DM-domains are highly conserved both in their structural features and in their involvement in sex differentiation in taxa as distant as nematodes, insects and vertebrates. In fact, the homologues of dmrt1 in *Caenorhabditis elegans* (MAB-3) and *Drosophila melanogaster* (DSX) are important factors located downstream in the sex determination cascade (Raymond et al. 1998), and in vertebrates, the DM-transcription factor-1 (DMRT1) is involved in male sexual differentiation in teleosts, reptiles, and mammals (Smith et al., 1999). In birds, DMRT1 is present on the Z chromosome but absent from the W and is considered the best candidate for the dose-dependent master sex determiner in this group (Shan et al. 2000; Smith et al. 1999, 2003). In humans, DMRT1 is located downstream in the sex determination pathway and a deletion of one copy is associated with XY male to female sex reversal (Raymond et al. 1999). Nanda et al. (2002) showed that the sex determining locus dmrt1bY in medaka resulted from a duplication of an autosomal dmrt1 gene. The duplication event followed by insertion of the chromosomal segment into another chromosome, is very recent. In fact, only the closely related species, *O. curvinotus*, has dmrt1bY on a homologous Y chromosome, but the gene was not found in any other *Oryzias* species (Kondo et al. 2003). Interestingly, in the sister species of *O. curvinotus* (*O. luzonensis*), dmrt1bY is absent, suggesting the following scenario: dmrt1bY arose recently as the new master sex determiner in the common ancestor of *O. latipes*, *O. curvinotus* and *O. luzonensis* but in the latter species it was secondarily lost. Evidence that this young sex determiner might not "be there to stay" is suggested by the relatively high frequency of XX males in laboratory strains: these are lacking dmrt1bY, showing that the gene is not indispensable for male development. Given this instability, modifier genes might take over the control of sex determination leading to the death of dmrt1bY in related species. Phylogenetic analyses based on mtDNA allow dating of the rise of dmrt1bY, which must have occurred in the common ancestor of those three *Oryzias* species. Their lineage separated 10 million years ago from *O. mekongensis*, hence the duplication event leading to the establishment of dmrt1bY as master sex determiner must have the same age: this places dmrt1bY as the youngest vertebrate Y known (the age of the mammalian Y chromosome being estimated at around 170-210 million years). Medaka's Y chromosome degeneration is strictly limited to the Y-specific region, outside which the chromosome is fully recombining with the exclusion of a 30 cM region in the vicinity of the Y-specific region. Although this has degenerated so that dmrt1bY is the only functional gene included, the progressive reduction of the pseudo-autosomal region predicted by the classical view of sex chromosome evolution (Charlesworth 2000) has not taken place significantly in medaka's Y. This is due to the presence of two almost identical segments flanking the Y-specific region that have a copy on the X chromosome. Given the high degree of homology of X and Y, the two pair for most of their length and either Y-copy of the flanking segment can pair with the X copy allowing crossover to take place, with the other Y-copy left looping out. This 'intragenic crossovers of the X copy with both Y copies' (Kondo et al. 2006) therefore might protect against degeneration outside the Y-specific region. This might account for the striking differences in the extension of chromosome degeneration between the medaka Y and the stickleback Y, of similar age and even the much younger papaya sex chromosome, the latter two having large sequence divergence over a large distance from the sex determining region.

The genesis of the medaka dmrt1bY as well as the evolution of the stickleback and the salmonid sex determining systems, suggest that the traditional pathway of sex chromosome evolution might not be in fact the most common. A short chromosomal segment containing a gene involved in sex differentiation (in an autosome, as in medaka) or containing the master sex determiner (in a sex chromosome, as in salmonids) can be moved, either by direct chromosomal rearrangements or following duplication, to a new chromosome assuming a new role as master sex determiner and will be automatically isolated from recombination with the X. Twin identical sequences flanking the Y-specific region and alternatively recombining with an homologue on the X as in the medaka or palindrome sequences on the Y as in mammals might counteract the forces that were theoretically identified as leading to the eventual demise of a Y chromosome (Charlesworth, 2000).

This incursion in the proximate mechanisms behind sex determination in fishes reveals the potential of studies employing teleost systems to unveil both the basic themes and the extraordinary diversity of sex determination in nature.

Back to the origins: Poeciliid sex determination

Some of the first pioneering studies on the inheritance of sex in animals involved poeciliid fish and were made possible by the many sex-linked colour genes available (Aida, 1921; Gordon, 1927; Winge, 1930). As a result, the family Poeciliidae has been thoroughly studied with respect to sex determination. However, the sex determining gene of any species is yet to be discovered. With one exception, most species lack heteromorphic sex chromosomes, suggesting a general young age of the sex chromosome system or, possibly, frequent transitions to new sex determining systems. Only in the western mosquitofish *Gambusia (affinis) affinis*, which shows female heterogamety, the W chromosome is large and cytologically well differentiated. But interestingly, in the sister species, the eastern mosquitofish *G. (affinis) holbrooki*, male heterogamety has evolved and sex chromosomes are homomorphic (Black & Howell, 1979; Angus, 1989).

In the guppy (*Poecilia reticulata*) and in some mollies (*Poecilia velifera, Poecilia latipinna*) a XX/XY system is in place but there is evidence for male and female autosomal sex determiners, leading to the generation of XX males and XY females (Winge 1930). In *Poecilia sphenops* both male and female heterogamety are reported (Schröder, 1964). The swordtail *Xiphophorus helleri* has a complex sex determining system: some authors (Peters, 1964; Bull, 1983) have suggested that *X. helleri* has a polyfactorial system with multiple male and female sex determining factors interacting, but others (Kallman 1984; Kallman & Bao, 1987) have proposed that numerous autosomal modifiers with many alleles of different strength interact with a standard monofactorial gonosomal system.

I will now consider in more detail the 3-factor sex determining system that characterises both the platyfish *Xiphophorus maculatus*, long the subject of empirical and theoretical research, and the Lake Victoria cichlid *Neochromis omnicaeruleus*, studied in this thesis.

The platyfish has three distinct sex chromosomes (X, Y, W) with the W chromosome absent from most populations. XX, WX and WY genotypes are females, while XY and YY are males. WW individuals are viable females, and can be obtained in the lab but appear to be absent or very rare in the wild.

Kallman (1984) proposed that male determining genes are present in all three sex chromosomes. Only the Y allele is active, while the W and X alleles are suppressed by autosomal repressors. The W chromosome has a Y specific repressor of the male determining allele, explaining WY females.

Bull (1983) analysed multiple factor systems such as the platyfish's and showed that an infinity of equilibrium genotype frequencies exists with 1:1 sex ratio, defining a continuous surface connecting alternative 2-factor sex determining systems. Interestingly, this result seems general for any number of factors. Bull explored also the implications of including fitness differences but limited his analysis to simple multifactorial systems such as the 3-factor platyfish system. He observed that when WX had higher fitness than the other female genotypes (XX, WY), the polymorphism was maintained, but male or female heterogamety evolved from the polymorphic state when WX had lower fitness. Similarly, when YY had lower fitness than XY, the invasion of W was precluded since it would lead to the production of less fit males (YY).

While X and Y recombine on most of their length and carry many colour genes, the W chromosome has no colour genes and in fact does not share any of the known X/Y genes with the exception of the Xmrk proto-oncogene. Colour genes are likely to be costly sexually selected traits in the platyfish, as shown for many poeciliids (e.g. Endler 1980). Kallman (1970) suggested that W might have invaded a XX/XY system as a dominant female determiner thanks to its lack of colour genes. Females carrying colour genes would bear the costs of increased predation and males lacking them would be rejected by females. But a sex chromosome lacking colour genes would invade if it is limited to females. However the origin and maintenance of such a polymorphism in sex determining factors is crucially affected by the fitness of all sex chromosomes and their epistatic effects in different genotypes, as pointed out by Bull (1983). The author showed that Kallman's hypothesis does not hold if colour genes are Y-linked. In that case, W would not invade as WY females would be selected against. If there is recombination between X and Y so that a similar number of colour genes is on X and Y (but not on W), W might invade as predicted by Kallman. However, colour genes are likely to be antagonistic (advantageous to males, detrimental to females) and therefore will tend to accumulate on the Y chromosome. For this reason, Bull (1983) concluded that equal frequencies of colour genes on X and Y would be unlikely and that only a modelling approach would allow to infer possible invasion pathways for W, when fitness of the sex factors is included.

Sex determination in cichlids and the case of Neochromis omnicaeruleus

Tilapia species (Cichlidae) have relatively undifferentiated sex chromosomes, a hallmark of early stages of sex chromosome evolution. Both male and female heterogametic species are present. Moreover, studies on hormonally sex reversed individuals, gynogenetic and androgenetic strains, and natural populations, have highlighted the fact that, together with a prevalently monofactorial system (Wolfarth & Wedekind, 1991), secondary autosomal genes with only partial penetrance (Mair et al. 1991; Sarder et al. 1999) as well as environmental factors such as temperature (Baroiller et al. 1995; Abucay et al. 1999), contribute to define sex determination in these species. Although no major cytological differences are apparent in the sex chromosomes of any tilapia species, by using synaptonemal complex analysis, incomplete pairing was observed in the homologous terminal region of the longest bivalent in *Oreochromis niloticus* (Carrasco et al. 1999) and in *Oreochromis mossambicus* (Campos-Ramos et al. 2003). These species are considered to be male heterogametic (Jalabert et al. 1974; Chen 1969). In the female heterogametic *Oreochromis aureus*, two unpaired regions were identified, one being homologous to the *O. niloticus* and *O. mossambicus* longest bivalent, and a second region on a distinct bivalent (Campos-Ramos et al 2001). By bulked segregant analysis, Lee et al (2003) were able to identify microsatellite markers linked to the XY locus on LG1 in *O. niloticus*, and Lee et al (2004) found markers linked to two independently segregating sex loci on LG1 and LG3, in *O. aureus*. Interestingly, in this species, the dominant male determiner on LG1 (XY sex locus) is homologous to the *O. niloticus* locus on LG1. The dominant female determiner on LG3 (WZ sex locus) is epistatic on the male locus on LG1. A possible breakthrough is the recent work of Shirak et al. (2006) which attempted to map eleven genes with key roles in sex determination in other vertebrates on an *O. niloticus* male x *O. aureus* female F2 hybrid. The positions of two of these genes on linkage group 23, Dmrta2 and Amh, overlapped with QTL previously associated with sex determination in tilapias and with a QTL region for sex-specific mortality, which might identify a region of accumulation of deleterious alleles in proximity to the sex determining locus. The authors conclude that Dmrta2 and Amh might represent the putative master sex determiners in tilapia. It must be noted that members of the DMRT class are widespread regulators of sex determination in both invertebrates and vertebrate classes and in one fish, the medaka, as discussed above, one dmrt gene has raised to the role of master sex determiner. Amh is involved in sexual differentiation in mammals and birds but most of all, it is suspected to be a direct negative regulator of the aromatase gene, which converts testosterone to estradiol-17h, necessary for oocyte growth. Since aromatase is thought to be central in the process of environmental sex determination, Amh might mediate the influence of temperature on sex determination in tilapia species. However, it is unclear whether either of these two genes is the master sex determiner, as suggested by Shirak et al. (2006). It is possible that Dmrta2 and Amh might simply represent minor sex factors that interact with major determiners on LG1 (and LG3 in *O. aureus*) occupying a downstream position in the sex determination cascade.

While intense research is devoted to members of the tilapiine cichlids, given the economic relevance of sex control for aquacultural purposes, scarce information is available on the sex determining system of other cichlids.

In the neotropical Midas cichlid (*Cichlasoma citrinellum*), Francis & Barlow (1993) suggested that the relative size at the juvenile stage was sufficient to induce differentiation of the sexes. A case of pH influence on sex determination was reported for three West African species, *Pelvicachromis pulcher*, *P. subocellatus* and *P. taeniatus*, by Rubin (1985). In the *Apistogramma* species complex, there is evidence for temperature and pH as major determinants of sex at maturity (Romer & Beisenherz, 1996), but in the same study, the African mouthbrooder *Pseudocrenilabrus multicolor victoriae* did not show any sign of environmental influences on sex determination.

Finally, a suspected case of hermaphroditism was reported for the Neotropical *Crenicara punctulata* (Carruth, 2000).

One study on the Neotropical cichlid *Geophagus brasiliensis* (Michele & Takahashi, 1977) found evidence of differentiated sex chromosomes, but Post (1965) did not find any evidence for the East African *Lamprologus leleupi.* Only two caryological studies have been performed so far on haplochromine cichlids (Kornfield et al 1979; Thomson, 1981) both not detecting any sex chromosomes in the species *Haplochromis flavijosephi, Melanochromis auratus, Astatotilapia burtoni.*

Recent work on the Lake Victoria haplochromine cichlid *Neochromis omnicaeruleus* by Seehausen and his collaborators (1999) and in the *Metriaclima* (*Pseudotropheus*) *zebra* species complex by Holzberg (1978) and Knight (1999) have revealed a common complex genetic system underlying a W-linked colour pattern. In both cases, experimental crosses and frequency distribution of phenotype classes in the wild suggest the presence of a 3-factor (*sensu* Bull, 1983) sex determining system (X, Y, W) interacting with autosomal repressors of W. Maintenance of polymorphism in sex determination is rare and is dependent on the fitness values of each sex factor (Bull, 1983). These equilibria allow the dissection of the complex interactions normally leading to the fixation of a particular sex determining system and provide testable hypotheses for the evolution of alternative heterogametic systems, as in studies of platyfish sex determination. This polymorphism in sex determining factors associated with W-linkage of colour genes might be relatively common in the rapidly radiating cichlids of Lakes Victoria, Malawi and Kivu.

Part II

Sex linkage: sexual selection, sexual antagonism and sex reversal

Sex chromosome evolution is characterised by an increase in the frequency of transpositions, duplications, deletions as well as large chromosomal rearrangements, as part of the degeneration process (Charlesworth et al. 1994; Charlesworth & Charlesworth, 2000; Marshall Graves, 2006). This, in turn, determines unusually high levels of genetic variability in sex-linked traits. For example, in the platyfish (*Xiphophorus maculatus*) there is extreme variation at sex-linked genes such as colour loci, the pituitary P locus, the Mdl and ONC-Xmrk loci responsible for melanoma formation, etc. (Froschauer et al. 2001; Volff & Schartl, 2001). Given the accumulation of sex related genes on the sex chromosomes, the rapid generation within and/or between populations of polymorphism in these traits, as a consequence of frequent rearrangements on sex chromosomes, might help explain the role of sex-linked genes in both pre- and post-zygotic isolation.

A large body of work has been devoted to explore the effects of sex linkage on postmating reproductive isolation since the famous observation of J.B.S. Haldane that "when in the offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous" (Haldane, 1922). Although Haldane's rule appears to be one of the strongest "laws" in evolutionary biology, holding for 151 out of 157 crosses in male heterogametic taxa (mainly Drosophila species and mammals) and 91 out of 98 crosses in female heterogametic taxa (butterflies and birds), its causes are likely to be diverse (Hollocher & Wu, 1996). The dominance theory (Muller, 1940), the faster male theory (Wu & Davis 1993) and the faster X theory (Charlesworth et al. 1987; Coyne & Orr, 1989) are today considered the most likely explanations, with the latter two hypotheses contributing in male heterogametic taxa to an effect that is probably principally due to dominance effects, i.e. the (at least partial) recessivity of the alleles causing hybrid inviability and/or infertility. This also implies that genes involved in speciation by postmating processes should be at least partially recessive (Orr, 1997).

Here I would like to concentrate instead on the possible effects of sex linkage on behavioural speciation (Ritchie & Phillips, 1998; Coyne & Orr, 2004).

It has been pointed out that reduced recombination on the sex chromosomes might facilitate the build-up of adaptive gene complexes between sex-linked traits under natural or sexual selection and promote ecological or sexual divergence (Sperling, 2002). Reviewing the literature on the genetics of sexual isolation, Ritchie and Phillips (1998) suggest three parameters affecting the incidence of sex linkage on signals and preferences responsible for species divergence. The authors point out that i. sexual selection is in general more intense on males, i.e. shifts in sexual traits are more deleterious to males than to females; ii. mutant signals and preferences are subject to 'unequal selection pressures': preference distributions (the 'response window') are generally wider than signal distributions. As a consequence, a shift in the signal will correspond to a small deviation from the optimum preference but a shift in the preference will cause a large deviation from the signal optimum, so that the selective pressure against shifts in preferences will be stronger than selection against shifts in signals. Finally, iii. sex linkage favours the accumulation of favourable recessive mutations that are immediately exposed to selection in the hemizygous sex. On the basis of these three elements, Ritchie and Phillips (1998) conclude that the likelihood of linkage of traits involved in sexual isolation is highest for female heterogametic systems in which females are the signaller and the signal is sex-linked. In other words, a shift in the sexual signalling system generating premating isolation is most likely in ZZ/ZW systems with W-linked sexually selected trait, as frequently occurring in butterflies (Prowell, 1998; Ritchie and Phillips, 1998).

Interestingly this is exactly the case in *Neochromis omnicaeruleus* and in the models of Lande et al. (2001), where males are the signal receiver (male mating preferences) and females are the signaller with the signal being W-linked. This suggests that such pattern of signal and preferences might be particularly prone to sexual isolation by shifts in the sexual signalling system.

Another interesting prediction of Ritchie and Phillips (1998) based on their analysis of signal response windows is that sexual signals might be more likely to be oligogenic as mutations with large effects might be, in the authors' words, "more likely to be tolerated" while the coordination of the sexual signalling system by gradual fine re-tuning of preferences on the shifted signal would be best accomplished by polygenic inheritance of preference. However, whether preference will have oligogenic or polygenic inheritance might depend also on the mode of communication involved: major genes for preference (and for the signal, too) might be expected for chemical and, possibly, acoustic communication while visual communication might involve more complex neural processing and therefore a polygenic action.

There is growing evidence for a biased distribution of genes controlling sex-related functions, sexually selected traits, mating preferences and sexually antagonistic traits on the sex chromosomes. Reinhold (1998) reviewed the available data on reciprocal crosses in insects and mammals (though most data were on *Drosophila* and mouse), and found evidence for a strong X-effect on sexually selected traits (more than 30% influenced by X-linked loci) and no influence of X-linkage on non-sexually selected traits. Sex linkage of sexually selected traits has been found mainly in insects, a bias that might not reflect an effective preponderance of sex linkage on sexual traits in this group but rather a research bias: beside *Drosophila* (Reinhold, 1998), sex-linked sexually selected traits are known in *Colias* butterflies (wing colour patterns: Silberglied & Taylor, 1978) in the grasshoppers *Corthippus biguttulus* and *C. mollis* (song: von Helversen & von Helversen, 1975), in the bushcricket *Ephipigger ephipigger* (song: Ritchie, 1996) and in the chrysomelid *Gonioctena variabilis* (de Zulueta 1925; Galán 1931). Guppies and poeciliids in general, provide various examples of Y-linkage of colour genes affecting males' conspicuous colouration under female choice (Fisher 1930; Lindholm & Breden, 2002; Lindholm et al. 2004). Male pigmentation in the medaka *Oryzias latipes* (Wada et al. 1998) and male plumage traits in *Ficedula* flycatchers (Saetre et al. 2003) are X-linked. A black blotched colour pattern, common in the cichlid species flocks of Lakes Victoria and Malawi, has been shown to be W-linked and under mate choice in the species *Neochromis omnicaeruleus* from lake Victoria and *Metriaclima zebra* gold, from Lake Malawi (Seehausen et al. 1999; Knight 1999; this thesis).

Information on sex linkage of mating preferences is much less abundant: Z-linked mating preferences have been found in some races of the bushcricket *Ephipigger ephipigger* (Ritchie 1996), in *Colias* butterflies (Grula & Taylor, 1980) and in *Utetheisa* moths (Iyengar et al 2002). Inspired by the case of *Utetheisa*, Reeve & Pfennig (2003) and Iyengar et al (2002) applied their 'protected invasion theory', developed in the context of eusociality in haplodiploid vs diploid insect species (Reeve & Shellman-Reeve, 1997), to suggest that ZW/ZZ heterogametic systems might be more conducive to sexual selection than XX/XY systems. The authors showed that new alleles for male trait or female preference are less likely to be lost by chance when rare in species with female heterogamety. Z-linkage of trait and preference in the parents leads to all sons inheriting the preference from the mother and half of them inheriting the rare trait from their father. This particular configuration, compared to X linkage or autosomal location of trait and/or preference, grants the highest number (50%) of offspring carrying both the new trait and the new preference alleles, and therefore, the lowest risk of random loss, when rare (Reeve & Pfennig, 2003). The authors suggest that this asymmetry between male and female heterogamety might explain why extreme ornaments are prevalent in female heterogametic systems (e.g. birds, butterflies) while male heterogametic systems (e.g. mammals) tend to develop armaments over ornaments.

An earlier work by Hastings (1994) had arrived to a similar conclusion by recognising the essentially antagonistic nature of many sexually selected traits. Hastings developed a model for the evolution of female mating preferences for a male handicap with male-limited expression. The author observed that when preferences for a male-limited trait are W-linked, they can spread even when heavily deleterious to males and new alleles for more extreme preferences can invade as long as they are favoured in females. On the contrary, choice genes on autosomes or on X chromosomes receive the benefits of being associated with the fitness alleles signalled by the handicap but they also pay the costs of being associated with the genes for the handicap. Recently, Kirkpatrick and Hall (2004) developed more comprehensive models of the effects of sex-linkage on the operation of sexual selection for indirect benefits, under either Fisherian runaway or a good genes process. They found that Z-linked female preference and either autosomal or Z-linked male trait were particularly conducive to a runaway process. With X-linked female preference, X-linked or autosomal male trait favoured a good gene scenario. W-linkage of female preference with Z-linked or autosomal male trait, again, favoured good genes sexual selection. When female preference was autosomal, a Z-linked male trait was conducive to a good genes process. Y-linkage of display does not allow the build up of a genetic correlation between trait and preference, making the runaway and the good genes scenarios impossible. Finally, sex linkage can facilitate the build-up of positive feedbacks (Crespi, 2004) between genes involved in pre-zygotic and post-zygotic isolation. When the two types of genes are in physical linkage, mate choice leads to an increase in frequency of genes for postzygotic isolation; these, in turn, will generate stronger pre-zygotic barriers, in a positive loop accelerating the establishment of reproductive isolation (Servedio & Saetre, 2003). Hall & Kirkpatrick (2006) modelled the effects of sex linkage for the evolution of reinforcement of mate preferences on an island. By using a weak selection model based on the quasi-linkage equilibrium of Barton & Turelli (1991), the authors showed that reinforcement can evolve even when intrinsic hybrid incompatibilities are absent, as a consequence of suboptimal values of the male sexually selected trait in hybrids. Moreover, Hall & Kirkpatrick's (2006) analysis revealed a strong effect of sex linkage of mating preferences on the strength of reinforcement. In particular, the highest levels of reinforcement were obtained when the trait was autosomal and the preference X-linked or Z-linked. Autosomal preferences and X-linked traits or X-linked preference and Y-linked traits were particularly adverse for the evolution of reinforcement. Lemmon & Kirkpatrick (2006), using the same modelling approach, analysed in detail the effects of sex linkage for reinforcement driven by hybrid incompatibilities. Their results showed that with X-linked (or Z-linked) inheritance of female preferences, X(Z)-linked incompatibilities had a stronger effect on reinforcement than autosomal incompatibilities; however, when female preferences were not sex-linked, autosomal incompatibilities contributed more to reinforcement than X-linked ones. Regardless of female preference inheritance, autosomal-X (and autosomal-Z) incompatibilities were favourable to reinforcement but Y-linked incompatibilities contributed only very little to it.

Hall & Kirkpatrick (2006) conclude that "we should never see W-linked preferences playing a role in reinforcement". Probably the best taxonomic group in which to test the authors' prediction might be the Lepidoptera, in which females are heterogametic, there is evidence of X-linkage of both sexually selected traits and female mating preferences (Prowell, 1998) and instances of reinforcement have been found (Luchtanov et al. 2005).

In the light of Seehausen et al.(1999) empirical work on the cichlid species *Neochromis omnicaeruleus* and Lande et al. (2001)'s models of speciation by selection on female-linked sex determining factors, it would be interesting to use Hall & Kirkpatrick (2006)'s and Lemmon & Kirkpatrick (2006)'s modelling framework to explore the consequences of sex linkage of female traits and/or male mating preferences on the potential for reinforcement. This approach might also allow investigating the consequences of sex linkage in a scenario of secondary contact and reinforcement between two populations with opposing heterogametic sex and male mating preferences for a female-linked trait.

Consequences of sex reversal for sexually antagonistic and sex-limited sexually selected traits

So far, we have seen that there are good theoretical reasons to expect sexually antagonistic genes, sexually selected genes and generally genes involved in reproduction to be located on the sex chromosomes. The experimental evidence for this effect is growing and more will probably be soon available with the proliferation of genomic map projects for many model systems. Theoretically, rapid reversals of the sex determining systems are possible, either driven by selection on the sex determiner or on tightly linked loci or by a combination of genetic drift and sex ratio selection, in the absence of an intrinsic fitness advantage on the sex determiner or the locus tightly linked to it. Moreover, the brief review on the stunning variety of sex determining systems in teleost fishes suggests that changes in sex determination are not only possible but probably frequent. So if reversals are possible in theory and might be even frequent in natural systems, what happens to genes with a biased genomic distribution, i.e. sexually antagonistic genes and, at least in part, sexually selected traits, when a reversal such as envisaged in Bull's (1983), Lande et al.'s (2001) or Vuilleumier et al's (2007) models takes place?

Below, I attempt to explore some of the possible consequences of a reversal in sex determining system on the genomic distribution, and eventually on the fate, of sexually selected traits and sexually antagonistic traits. I will try to argue that the differences in fitness values of such traits in alternative heterogametic systems affect not only their location (or re-location) and in general their future in that genome but also the likelihood of a particular transition from one system of heterogamety to the other.

1) *Sex linkage of sexually antagonistic traits might favour or oppose sex reversal e.g. favour or oppose the invasion of a dominant sex determiner that reverses the heterogametic system.*

In this section, I argue that transitions between heterogametic systems can resolve intersexual ontogenetic conflict (Rice & Chippindale 2001) arising by the accumulation of sexually antagonistic genes on the sex chromosomes, making such transitions favoured by selection. Other transitions will generate such type of conflicts because of the expression of antagonistic genes in the "wrong" sex. Therefore these transitions will be opposed by selection acting on those antagonistic genes.

Recalling Rice's (1984) predictions on the invasion of antagonistic genes on the sex chromosomes, I look at the consequences of a reversal in the sex determination, such as in the simple models of Bull (1983) and, as in Lande et al. (2001), I assume that reversal does not involve the origin of new sex chromosomes from autosomes, i.e. new sex factors epistatic on the old master sex determiner arise on the same sex chromosomes.

Under these assumptions, in a male heterogametic system XX/XY, a reversal leading to a female heterogametic XY/YY (ZW/ZZ) system will be favoured by dominant female advantageous antagonistic traits accumulating on the X. This is because after sex reversal the traits will become female-limited on a neo-W chromosome. In this way, reversal of the heterogametic system leads to a resolution of intragenomic conflict. In a similar way, in a ZW/ZZ system, the reversal to a male heterogametic system is favoured by dominant male advantageous antagonistic traits accumulating on the Z chromosome, because this leads to the resolution of sexual conflict.

On the contrary, recessive female advantageous-male deleterious alleles accumulating on the Z chromosome and recessive male advantageous-female deleterious alleles on the X chromosome will oppose a reversal of the sex determining system because this would lead to their sex-limited expression in the sex in which they are deleterious (e.g. a recessive male advantageous female deleterious allele on X would find itself female-limited on W).

Male antagonistic alleles on Y and female antagonistic alleles on W can also oppose a sex reversal although the effect is weaker and will depend on their dominance. For example, a female antagonistic gene on W, after reversal, would be expressed in both sexes but if dominant, it would still be expressed 2/3 of its time in females and therefore its location on the sex chromosome still advantageous compared to an autosomal location.

The effect of sex linkage on the likelihood of a particular transition (a reversal of heterogamety) will eventually depend on the number of sexually antagonistic traits, their location and the size of their fitness effects. It is also possible to envisage frequent reversals of heterogamety fuelled by the invasion of dominant male advantageous female deleterious genes on Z and dominant female advantageous male deleterious genes on X.

Lande et al.'s (2001) models of speciation examine the invasion of a dominant X-linked female determiner, followed by the invasion of a colour gene physically linked to this new sex determiner. I will describe the model in a following paragraph and there I will propose that the opposite sequence of events (first an X-linked colour gene, then a dominant sex determiner on X) might in fact be not only possible but might resolve some difficulties of the original model.

Even in the case of the rise of a new sex determiner on an autosome (the rise of a neo-sex chromosome) the general conclusion that sexually antagonistic traits can oppose sex reversal is not compromised. As observed before, when a new master switch sex determiner evolves, the old sex chromosome can either be lost or rapidly eliminate repetitive sequences and re-gain functionality, as in the old sex chromosome of *Drosophila miranda*, where neo-sex chromosomes have recently evolved (Yi & Charlesworth, 2000). Given the intense traffic between sex chromosomes and autosomes, it is expected that eventually, sexually antagonistic traits exposed to expression in both sexes by the rise of a new sex chromosome will be relocated (copied, retrotransposed, etc). However, this process will take time during which selection will act against these genes when expressed in the "wrong" sex, and possibly drive them to extinction before genomic relocation has rescued them from selection against them.

2) A reversal in the heterogametic system might change the likelihood for the evolution of sexual selection by good genes or Fisherian runaway.

This stems from Kirkpatrick and Hall's (2004) analysis of the effects of sex linkage of male trait and female preference on the generation of sexual selection. Here we move the focus from sexually antagonistic traits to sexually selected traits with sex limited expression (sexual trait expressed only in males, mating preference for the trait expressed only in females). A reversal in the heterogametic sex changes the genomic location of male sexually selected traits and female mating preferences. Since different combinations of genomic locations of trait and preference are more or less conducive to the operation of sexual selection by good genes and/or runaway processes, as demonstrated by Kirkpatrick & Hall (2004), sex reversal can affect the probability that sexual selection will evolve. Therefore, as a consequence of the relocation of trait and preference following reversal, a change in heterogametic system might trigger a burst of sexual selection. Conversely, sex reversal might determine the loss of the genetic correlation required for both models of indirect sexual selection and oppose the evolution or the maintenance of a sexual selection regime in a population. Finally, a population incurring into sex reversal might differentiate itself from other populations and possibly evolve behavioural isolation as a consequence of a favourable stage, set by sex reversal, for the evolution of either runaway or good genes sexual selection.

In particular:

i. if sex reversal affects a system with Y-linked male sexually selected traits and female preferences located on an autosome or X-linked, sex reversal will lead to a situation in which the trait is Z-linked and the preference, respectively, autosomal or W-linked. This is particularly favourable to the establishment of sexual selection by good genes, starting from a condition (Y-linkage of trait) that does not allow the build up of a correlation between trait and preference necessary for either mode of indirect sexual selection.

ii. X-linkage of preference and autosomal location of the trait favours the establishment of good genes sexual selection: sex reversal leads to a similarly favourable condition, W-linkage of preference and trait autosomal. Although Kirkpatrick & Hall's (2004) modelling considers W-linkage to be superior to X-linkage for the evolution of good genes sexual selection, the difference in value between the two is marginal. Sex reversal in either direction will lead to a system with strong sexual selection, the two conditions equally favourable for the evolution of the genetic correlations between male trait and female preference required for a good genes process.

iii. Sex reversal represents a dead end for all other combinations of trait and preference, because it leads to the male trait being confined to females or the mating preference confined to males. Contrary to the re-location of sexually antagonistic traits after sex reversal (hypothesis 1), here we are dealing (in line with Kirkpatrick & Hall's assumptions) with sex limited traits. In other words, a male trait here is not expressed in females and the same is true for a female preference which is silenced in males. As a consequence, if sex reversal moves a male trait on a W chromosome or a female preference on the Y, the trait will not be expressed and there will be no direct deleterious consequences for the individual. It is then possible that the "misplaced" character might be lost from the population. This fact is interesting for two reasons: first, if sex reversal leads to the loss of the expression of a character originally favouring its carrier (e.g. a sexual ornament in a male), the sex reversal genes might be selected against and fail to invade; sex reversal would be impeded. Second, populations might experience changes in environmental conditions, either as shifts in habitat characteristics (e.g. light spectrum, turbidity, temperature, water flow, vegetation coverage, etc) or as shifts in population density or in the predator community, etc. All these can potentially affect the fitness value of a sexually selected trait (or a female preference) and populations will differ in the intensity of natural and sexual selection affecting the character. As a consequence, if for example a sex-linked conspicuous male ornament becomes suddenly highly deleterious following the invasion of a visual predator, the

invasion of sex reversal genes might be favoured, as sex reversal would provide a fast solution to the problem of eliminating (= preventing the expression of) the deleterious character.
In the discussion above the main point was that the invasion of sex determiners can change the linkage patterns of sex-linked genes and most notably of sexually antagonistic genes in ways that can favour or oppose the reversal itself. In this framework it might be possible to extend this reasoning to the likelihood for the establishment of environmental sex determination (ESD) from a purely genetic system of determination (GSD). Linkage to the sex determining factor/s of sexually antagonistic traits might prevent or at least oppose the invasion of an environment-sensitive sex determining factor. In fact, this would determine the expression of the antagonistic gene in the sex where it is detrimental, hence its invasion would be opposed by selection. Of course, if the advantages of ESD override the effects of the antagonistic trait/s, ESD might invade, but at least in the case of neutral invasion, predicted by some models (e.g. Morjan, 2002), ESD might be prevented by the presence of sex-linked antagonistic traits. In other words, the rapid accumulation of sexually antagonistic genes, in proximity to the master sex determining factor, sometimes might stabilise GSD from the invasion of ESD factors and canalise the evolution of a certain genetic sex determining system.

In conclusion, environmental parameters, demographic forces, changes in mating strategies and any other factor likely to affect the local optimal population sex ratio might favour the establishment of new master sex determiners. The mode of sex determination might in turn dictate the fate and genomic location of traits under sexual selection or sexually antagonistic selection. On the other hand, in species with a recently established sex determining system, sex-linked traits might impose selection on sex determining factors, leading to transitions either between polymorphic states (multiple factor sex determination, Bull, 1983) and single factor sex determination or transitions between male and female heterogamety. This would feedback into the genomic distribution of genes with differential fitness effects in males and females possibly triggering redistribution of genes after the rise of a new sex chromosome.

Sexual selection can affect sex chromosome evolution in other ways, too. Here I provide only one example, to highlight the need for a more integrated view of the interplay between sex ratio selection, sex determination and sexual selection.

Postmating sexual selection in the form of sperm competition can directly affect the evolution of a Y chromosome: it is known that in primates, the Y chromosome contains various male reproductive genes and particularly genes with important functions in sperm production (Kuroda-Kawaguchi et al. 2001). Sperm competition is known to affect testis size, sperm counts, semen coagulation, sperm viability and speed (Briskie et al. 1997; Birkhead & Møller, 1998). Deleterious mutations appearing on Y might be dragged to fixation by hitchhiking on genes coding for sperm characteristics. By comparing genes in the X-degenerate region of the Y chromosome (i.e. the region once identical to the X counterpart) of human and chimpanzee, Hughes et al. (2005) found that most genes had been conserved in humans since the divergence from the common ancestor but many had inactivating mutations in the chimpanzee. The authors suggested that such inactivation might have been driven by hitchhiking of deleterious mutations driven by strong sperm competition acting on Y-linked genes affecting sperm production in a species, the chimpanzee, in which promiscuity is rampant.

I suggest this hypothesis could be tested by performing gene-by-gene comparisons of Y chromosome degeneration in sister species of taxa such as mammals, and particularly rodents, for which monogamous/polygynous species pairs are known, e.g. prairie and montane voles, jumping rat species, etc., but also, of course, primates. Considering the vast genomic information available for various species in both groups, male heterogamety and the stability of the sex determining system (an important prerequisite for such a test), rodents and primates would probably represent ideal systems to test hypotheses on the interplay between Y degeneration and intensity of sperm competition.

Part III

Female ornaments, the direction of sexual selection and the evolution of male choice

The classic view on female ornamentation dates back to Darwin (1871) who first considered the possibility of pleiotropic expression in females of conspicuous traits selected in males. This correlated response hypothesis was later modelled by Lande (1980) and Lande &Arnold (1985) and supported by some experimental evidence (Muma & Weatherhead, 1989; Hill, 1993; Cuervo et al. 1996). Given this pleiotropic effect between the sexes, only strong selection for sex-limited expression can eventually break the genetic correlation and allow the evolution of sexual dimorphism, but this process is thought to be relatively slow (Lande 1980). One additional consequence of the genetic correlation between the sexes is that males choosing ornamented females might in fact be selecting not only for ornamented daughters but for ornamented sons, too.

Recent phylogenetic studies examining gains and losses of sexually selected traits in birds (New World blackbirds: Irwin, 1994; dubbling ducks: Omland, 1997; tanagers: Burns 1998) and phrynosomatid lizards (Wiens, 1999) have revealed that evolutionary changes in conspicuousness are more frequent in females than in males and in fact females are more frequently gaining rather than losing a conspicuous colouration. This suggests that female colouration can often be directly under selection rather than the result of pleiotropy between the sexes of traits selected in males.

Direct selection on female colouration can be driven by i. competition for limited resources, either mates (intrasexual selection) or feeding, nesting etc. resources, or ii. by male choice (intersexual selection).

Competition for male partners will arise when there is large variation in male quality or their availability is very low (Parker, 1983, Langmore et al. 1996). Female colouration might represent a badge of status in aggressive displays over foraging territories (Bleiweiss, 1985, 1992), nesting sites (Whittingham et al. 1997) or over access to male partners (Johnson, 1988; Trail, 1990). Heinsohn et al (2005) recently showed that the extreme sexual dichromatism of the parrot *Eclectus roratus* is not due to intersexual selection but to female-female competition for nesting sites, where the female spends up to 11 months a year. Female colouration is highly conspicuous against the canopy background where females display aggressively competing for breeding hollows. Male colouration is cryptic against the background of the canopy but conspicuous against tree trunks, where nest entrances are and most male competition takes place. In conclusion, in this species the selective forces shaping male and female conspicuous colouration differ between the sexes, female colouration generating from sex-specific resource competition, male colouration from intrasexual selection.

Female ornaments might also be under sexual selection by male choice, when there is large variation in female quality (Parker, 1983; Owens & Thompson, 1994). In lizards, female-limited ornaments are relatively frequent (Cooper & Greenberg, 1992) and in the striped plateau lizard (*Sceloporus virgatus*) orange throat patches represent a condition-dependent signal of overall quality, encompassing body condition, parasite load and average egg size (Weiss, 2006). In Northern cardinals (*Cardinalis cardinalis*) more intensely coloured females provide more parental care to their young (Linville et al 1998) and in pied flycatchers (*Ficedula hypoleuca*) female ornamentation is associated with parasite resistance (Potti & Merino, 1996). In female barn owls (*Tyto alba*) the amount of black spots on their plumage correlates with the level of antibody response (Roulin et al. 2000; Roulin, 2004). Redder female arctic charr have a lower parasite load than drabber ones (Skarstein & Folstad, 1996). However, evidence of male choice for female ornamentation is still scarce. LeBas et al (2003) showed that female empidid dance flies (*Rhamphomyia tarsata*) invest in an honest signal of fecundity in the form of enlarged pinnate leg scales; these female traits are also under nonlinear correlational sexual selection by male choice. In the non sex-role reversed two-spotted goby *Gobiusculus flavescens* males prefer orange-red spots on the belly in females (Amundsen & Forsgren, 2001).

So, when are males choosy? It has been long recognised that the direction of sexual selection can be inferred from the operational sex ratio (OSR), 'the ratio of sexually active males to receptive females' (Emlen & Oring, 1977). Johnstone et al. (1996), using an ESS approach, showed that although variance in quality is obviously an important determinant of choosiness, so is the time necessary to process matings and in fact the latter has a larger effect. When processing times and variation in quality are similar for both sexes, mutual mate choice is possible. Parker & Simmons (1996) arrive to similar conclusions regarding the importance of 'processing time' (which includes mating, parental care, gamete replenishing, etc) by considering the potential reproductive rate (PRR) of Clutton-Brock & Vincent (1991), a proximate of the operational sex ratio (OSR), defined as 'the maximum rate at which an individual of a given sex can produce offspring, averaged across all individuals and conditions'. Parker & Simmons suggest that this rate is simply the number of progeny produced divided by the "time out" i.e. the time required to process a reproductive event and hence not available for mate-acquisition ("time-in"). The authors could show that the direction of mating competition can be predicted by the adult sex ratio and the population mean value of 'time out' for each sex. Kvarnemo & Simmons (1999), however, pointed out that, together with the 'time out' as a determinant of OSR, other parameters contribute to the direction of sexual selection, such as the relative advantages of obtaining a high quality mate. Also, they stressed that competition for mates and choosiness should not be regarded as alternatives but the choosy sex might be the most competitive, too. The OSR will determine the direction of intrasexual competition but not necessarily intersexual selection, since the latter is affected not only by availability of partners (i.e. OSR) but also by variance in mate quality (Kvarnemo & Simmons, 1999; Simmons & Kvarnemo, 2006). Kokko & Monaghan (2001) criticised the OSR-PRR approach and showed that PRR cannot be regarded as a proximate of OSR. Moreover, that neither OSR nor PRR are sufficient to predict the direction of sexual selection and that the cost of a single breeding attempt directly influences the evolution of choosiness. The authors found that although a male-biased sex ratio generates more intense competition among males, the threshold value at which males are more competitive than females is not 1, in general. If the cost of breeding is higher in females than in males, these will still be the competing sex even when the sex ratio is heavily female-biased. Since intrasexual competition and choosiness are not necessarily interdependent, different are also the conditions for their evolution. Kokko & Monaghan (2001) identify four factors affecting the likelihood for the invasion of choosiness in one sex: high sex-specific mortality costs of breeding, high mate encounter rates, low sex-specific mortality during mate searching and variation in mate quality favour the evolution of choosiness in one sex.

Male mate choice should then be favoured when male mating investment (including mate searching time, energy investment in courtship, courtship mortality, parental care, etc) is high and when the variance in female quality is large. The size of the effect of variance in mate quality might be one of the reasons for the widespread occurrence of female choice but the rather limited presence of male choice. In fact, the most important factor in determining female quality is fecundity while female genetic quality might in general not be as important as male genetic quality at least in promiscuous systems. Moreover, advertising genetic quality (by development of honest signals) might impose very different costs to males and females. The energetic costs of producing female gametes might trade-off with the costs of producing an honest signal of genetic quality and males might prefer direct benefits (fecundity) to indirect ones advertised by female ornaments. This should severely limit the parameter space for the evolution of female sexual ornaments. Chenoweth et al. (2006) suggest that males should develop stabilizing mating preferences for female ornamentation to avoid females investing too many or too few resources in the honest displays.

Servedio & Lande (2006) model the evolution of male and mutual mate choice in systems with polygyny. The authors consider two loci for preference, one for male and one for female mating preferences each with two alleles (preference, no preference). Traits target of mating preferences are controlled by a male trait locus and a female trait locus, each with two alleles (unpreferred allele, preferred allele). Important assumptions of the model are that all females have equal mating success, regardless of male choice and that female choice is based on both the male trait and its courtship effort. Their analysis shows that male mating preferences for arbitrary female traits cannot be maintained, unless male preference is associated with an increase in courtship output. If all males have equal total courtship effort and females have equal mating success, males courting preferred females will suffer higher male-male competition than males choosing unpreferred females, providing males with no preference with a frequency-dependent advantage over choosy males. We will encounter again this frequency-dependent effect when considering Lande et al.'s (2001) models for the evolution of male preference polymorphism and speciation inspired by the *Neochromis omnicaeruleus* blotched polymorphisms. Male choice can evolve when males court more intensely preferred females or when the female trait is an indicator of quality (the preferred female trait is associated with higher fecundity, higher viability or good genes, as seen in the examples above), or when strong correlations (due to pleiotropy) in trait and preference between the sexes are present. Interestingly, pleiotropy on trait only is sufficient to maintain male preferences in combination with linkage disequilibrium resulting from males choosing females that prefer these males.

But does choosiness in one sex affect the likelihood of choosiness in the other sex?

Servedio & Lande (2006) find that, when male and female preference genes are distinct, the evolution of one is completely decoupled from the evolution of the other. Although using a very different approach, Kokko & Johnstone (2002) reached opposite conclusions: their models applying game theory concluded that the evolution of choosiness in one sex reduces the mating rate in the other and hence the likelihood for the generation of choosiness in that sex. The authors observed that such divergence of sexual strategies might be the main cause for the rarity of mutual mate choice in nature.

An alternative approach to the study of mutual mate choice, explored by Bergstrom & Real (2000), makes use of two-sided matching theory, a technique developed in the 1950's to generate optimal pairing between college applicants and colleges (Gale & Shapley, 1962). Inherent in this approach is the assumption that both male and female individuals can differ among themselves in their preferences. The authors' analysis reached the conclusion that stability of mutual pairings is possible under monogamy but hard to maintain in polygamous systems. They also showed that in mutual pairing what matters is not female rejection of male courtship (female control) but just the opposite: 'the component of choice conferring the major benefit is the power to choose the individuals to whom one displays, not the power to choose whether to accept or reject courtships' (Bergstrom & Real, 2000).

I have mentioned initially that Seehausen et al (1999), studying the colour polymorphic lake Victoria cichlid *Neochromis omnicaeruleus*, found evidence for male mating preferences for female colouration. Also, there was evidence for variation in sex determining factors suggesting a (X, Y, W) system with autosomal modifiers, and W-linkage of two blotched colour patterns, generating three colour morphs in the population.

I will now describe in more detail their study and finally present a model by Lande et al. (2001) that derives conditions under which the invasion of a dominant female determiner W in an X,Y system and W-linkage of a trait under mate choice, can lead to rapid sympatric speciation, under a set of specific assumptions.

Part IV

The pieces of the puzzle fall into place: Neochromis omnicaeruleus, a system with variation in sex determining factors, sex linkage of colour genes, and male and female mating preferences for colour morph patterns.

Seehausen *et al.* (1999) studied the ecology and the genetics of colour and sex determination in *Neochromis omnicaeruleus*, a member of the haplochromine cichlid flock of Lake Victoria. The authors explored the possibility that this colour polymorphic species might represent an incipient stage in speciation, driven by mating preferences for sex-linked colour patterns in the absence of pre-existing ecological divergence between morphs. In some *N. omnicaeruleus* populations, up to three male and ten female colour phenotypes can co-occur (fig. 1). The three common male and female colour morphs are: i. males are blue, females are yellow-brown with dark vertical bars (P morph); ii. males and females exhibit black blotches on a blueish (males) or white-yellow (females) background (WB morph); iii. males and females exhibit black blotches on a pink to orange background (OB morph). Intermediate phenotypes exist, but are rare. Morph frequencies vary between populations but a recent study (Maan et al., *in press*) on the same population (Makobe Island) originally sampled by Seehausen et al. (1999) shows that phenotype frequencies have remained stable over the last 10 years (sampling in 1992-93, 1995, 1996, 2002-03). Breeding experiments highlighted that phenotypes rare or extremely rare (i.e. intermediate blotched female phenotypes and blotched males, respectively) or possibly absent (black females, i.e. homozygous for blotch) in the Makobe Island population, are easily obtained in the lab. The authors suggested that such discrepancy might be due to non-random mating, generating the multimodal distribution of phenotype frequencies in the wild.

Lab crosses also revealed that blotch colouration is associated with dominant X-linked sex reversal genes. Male heterogamety is considered to be the ancestral condition in the haplochromine cichlids of Lakes Victoria and Malawi (Kornfield 1981). The X-linked sex reversal genes in *N. omnicaeruleus* probably represent dominant female determining genes overriding the masculinizing effects of the Y chromosome in males, so that XY (blotched) females are generated. Here I refer to sex chromosomes as the chromosomes carrying sex determining genes without any implication over their state of degeneration. From hereafter, I will use the following notation: X chromosomes carrying dominant female determiners (sex reversal genes *w*) will be noted as W chromosomes, and the superscript ' will be used to indicate carriers of blotched colouration. The notation X' will therefore represent an X chromosome lacking the sex reversal genes but carrying blotch colouration, a W' will denote an X chromosome carrying both a dominant female determiner *w* and blotch colouration. A, a subscripts refer to alleles at the W_A locus determining the black and white morph WB; B, b subscripts to alleles at the W_B locus giving origin to the orange-blotched morph OB (fig. 1).

Since crosses between WB blotched females and OB blotched males yield no homozygote blotched offspring, WB and OB must be alleles at different loci.

The existence of blotched males is explained by the presence of autosomal recessive suppressors (M) of the dominant feminising genes *w* that can "rescue" maleness in XY individuals restoring Y action and giving rise to blotched male phenotypes. The genotype of these males is then W'YMM with the introduced notation, i.e. carriers of i. dominant female determiners w on the X chromosome = W, ii. blotched colouration ', iii. male sex determiners on Y, and iv. autosomal recessive male rescue genes. M genes have a pleiotropic effect on blotch colouration, intensifying its expression; they also appear to be morph specific, i.e. M_A rescues only from the action of a w_A female determiner, M_B from a w_B, only. This complex genetic hypothesis readily explains offspring sex ratio distortion in crosses between plain males (XY) and blotched females (W'X), as these lead to the production of XX, W'X, W'Y female genotypes and XY male phenotypes. The observed between-family variation in offspring sex ratio in crosses between plain males and blotched females is due to the effects of M alleles: when both XY males and W'-

carrying females have some male rescue M alleles, some or all W'Y offspring will be males (W'Ymm, W'YMm females, W'YMM males) partially or completely rebalancing the sex ratio. The recombination rate between blotch colour and female determiners w was estimated as $r = 0.052$, a value which is much larger than some Y-linked genes in guppies and platyfish. Therefore the observed linkage between colour and sex can only be maintained by selection against recombinant individuals.

To test whether colour morphs were experiencing any ecological divergence, Seehausen et al. (1999) used 13 morphological measurements, known to underpin fine-scale differences in foraging ecology in haplochromine cichlids (Barel et al. 1977), but they did not find any differentiation in morphospace between colour morphs.

Mating preferences in Neochromis omnicaeruleus

Male and female mating preferences were tested in two-way choice experiments. Plain females from plain (P) parents were equally courted by plain males and blotched males but responded more to the courtship of plain ones. Blotched WB females were courted more by plain males from a WB mother (P_{WB} males) than by plain males from a plain mother (P_P males) and more by WB males than by P_P males. WB females were equally responsive to all three types of males. In conclusion, while P females have a mating preference for P males, WB females do not show mating preferences for male colouration.

Seehausen et al (1999) found also evidence for male mating preferences for female colour patterns in this species. In particular, P_{WB} males and WB males courted more WB females than P females, P_P males courted more often P females.

The authors suggested that this pattern of male and female preferences is paralleled in the OB system, on the basis of choice experiments between a wild OB female preferring plain over WB males but not discriminating between plain and OB males and on the basis of field underwater observations of plain males courting OB females and a 20 minute focal observation of a OB male being visited by various females of all three P, WB, and OB morph but courting only OB females.

The evidence from the mate choice experiments and the multimodal distribution of colour phenotypes in the wild, highlighting the rarity of intermediates, both suggest that non-random mating between colour morphs is taking place and that the *N. omnicaeruleus* system might represent an incipient stage in sympatric speciation. Complete restriction of gene flow between morphs is probably prevented by the rarity of blotched males in the wild. These are readily produced in the lab but rarely seen in the field, possibly because of more intense predation pressure on blotched than on plain males. A study on differential predation on females of P, WB, OB colour morphs (Maan et al., *in press*) showed that kingfishers significantly caught more blotched than plain individuals. Courting males perform complex and conspicuous displays that might particularly expose them to predation. Courting blotched males might receive more attacks by visual predators than plain ones. Selection against blotched males hinders the fixation of M and the evolution of female mating preferences for blotched males in blotched females and therefore full assortative mating between colour morphs is prevented.

We have seen that in a multifactorial sex determining system (X, Y, W), the presence of dominant female determiners w can generate female-biased offspring sex ratios and the production of YY unfit or inviable males (resulting from WY mothers x XY fathers). Seehausen et al. (1999) proposed that mating preferences in *N. omnicaeruleus* might have evolved in response to sex ratio selection, to avoid matings that produce female-biased clutches, and to natural selection against the production of unfit YY individuals.

Variations on a blotched theme

Knight (1999) studied blotch polymorphic species in the Lake Malawi cichlid radiation and performed breeding experiments aiming at testing whether blotch genes are associated to sex ratio distorters, as in the case of the Lake Victoria cichlid *N. omnicaeruleus*. Linkage of blotch colouration with dominant female determiners (sex ratio distorters) was found in the rockdwelling cichlid species *Maylandia zebra* and *Maylandia* 'zebra gold'. The genetic model developed in *N. omnicaeruleus* by Seehausen et al. (1999) was consistent with the author's results once an additional autosomal colour modifier was added to the original three-locus model. In fact, in the Lake Malawi *mbuna* flock, blotch polymorphic species have often more extreme phenotypes (completely orange, yellow or white or black) than Lake Victoria *mbipi.* The fact that these extreme forms represent a highly homozygous condition of the blotch polymorphism comes from crosses between such phenotypes and plain individuals, resulting in blotched F1 offspring, and by crosses between blotched individuals resulting in orange F1 offspring (Knight, 1999). It was proposed that *N. omnicaeruleus* represents an incipient stage in speciation and that the interruption of gene flow between the two blotched incipient species (WB, OB) and the ancestral plain (P) species is prevented by selection against blotched males. This, in turn, exerts strong selection against blotched females expressing a mating preference for blotched males. If this represents the major obstacle to divergence and speciation in blotch polymorphic species, we would expect that in its absence, speciation would occur generating sister species fixed for alternative colour (and sex factor) alleles. In the Lake Malawi cichlid *Pseudotropheus lombardoi,* all males are orange (highly conspicuous) but the species lives in deep waters, where predation against conspicuous (i.e. blotched) males might be relaxed. On the other hand, in the Victorian *Paralabidochromis chromogynos* and the malawiians *Pseudotropheus callainos* and *P. estherae* in Lake Malawi, that live in shallower waters, females are blotched only, but males are plain. It is possible that in these species, female-limited expression of blotch has evolved, releasing W-carrying males from selection by predation and making speciation possible (Seehausen, 1999).

The presence of highly homozygous forms of blotch, generating phenotypes homogeneously orange, white, yellow, black, poses a challenge to a reliable estimate of the number of species that might be harbouring blotch colour genes linked to dominant female determiners or possessing colour patterns of different origin. Since blotch mutations disrupt the typical melanin pattern of dark vertical bars or horizontal stripes, characterising most haplochromine cichlids, the absence of such patterns is a good indication of the possible presence of homozygote forms of blotch. However, only large painstaking breeding programs and/or the development of adequate markers might allow large scale screening of both Lake Victoria and Lake Malawi cichlid radiations for the presence of blotch and sex ratio distorting genes.

A first attempt towards the isolation of the genes responsible for blotched colouration has been recently made by Streelman et al. (2003). Combining QTL mapping with association scans in natural populations, the authors identified a single region of the genome associated with the OB colour pattern, flanked by two microsatellite markers. Comparative mapping with the takifugu and the human genome, identified possible candidate loci for OB. In particular, *Pax7*, closely related to *Pax3*, a gene encoding a transcription factor necessary for melanocyte development, and ORFs for forkhead genes, that in humans are implicated in melanogenesis repression, represent promising candidates. Streelman et al. (2003) make also a striking observation: the OB locus lies close to a QTL for tooth shape and a tandem array of three cone opsin genes. Given that ecological divergence in East African Lake cichlids has been attributed to rapid ecological divergence and sexual selection by visual cues, this finding suggests the presence of supergene adaptive complexes that might have played a crucial role in the unfolding of the cichlid adaptive radiations. Supergene complexes have been shown to be involved in the adaptive evolution and diversification of *Heliconius* butterflies (Joron et al. 2006).

Blotched speciation: Lande's models

The possibility that the *N. omnicaeruleus* colour and sex factor polymorphism might represent early stages in sympatric speciation led Lande et al. (2001) to develop theoretical models exploring the conditions under which sex reversal and sexual selection on W-linked colour genes can drive rapid sympatric speciation. The authors consider the invasion of a dominant female determiner tightly linked to the original female determiner x (creating a W chromosome) that changes XY males into females.

The authors identify pathways leading to sympatric speciation under two main restrictive assumptions: i. tight linkage of the dominant female determiner with a marker trait, allowing the evolution of male mating preferences for carriers of W (male choice for W-linked traits), ii. pre-existing male mating preferences against the marker trait. I will consider both assumptions, later in the discussion and I will experimentally test the assumption ii. in a natural population (chapter III).

Reviewing the evolution of sex determination, we have seen that dominant female determiners overriding the masculinizing effect of a Y chromosome are not uncommon (e.g. in lemmings) and that multiple sex factors (x, y, w) can coexist in the same population (e.g. in the platyfish). The presence of W will create WX and WY female individuals and, as a consequence, YY males, too. When the sex chromosomes are at an early stage of degeneration, and effective population sizes are large so that such mutations have not been fixed, YY and XY individuals might have similar fitness. In populations with small effective size, however, partially recessive mildly deleterious mutations will not be effectively purged and will eventually go to fixation (Kimura et al. 1963; Crow, 1993), resulting in loss of fitness in YY genotypes. This effect might be magnified when the degeneration of the Y chromosome creates regions of reduced recombination and therefore permanent heterozygosity of mutations (Muller, 1918; Charlesworth & Charlesworth, 2000).

When YY suffer no fitness disadvantage, the invasion of W and consequent appearance of YY genotypes would lead to rapid purge of deleterious mutations by natural selection. Lande et al. (2001) assume a fitness advantage to W (10% increase in fecundity compared to X) that drives the dominant female determiner rapidly to almost 50% in females, in this way reverting the heterogametic system (WY females YY males). As seen with Bull's (1983) models for the maintenance of polymorphism in a three factor system, single determination systems (either male or female heterogamety) are connected by lines of equilibria where the sex ratio remains practically unbiased. Then, a colour mutation is seeded in the population, at low frequencies, on the W chromosome (a marker trait for W, in analogy with the W-linked blotch mutation in *N. omnicaeruleus*). The authors reason that a colour mutation arising on Y or an autosome would be selected against by female choice on male nuptial colouration. Being female-limited, the mutation is not exposed to sexual selection by females. Moreover, Lande et al. (2001) assume that all females mate and are equally fecund so that their preferences are under neither natural nor sexual selection. Since most males in the population exhibit pre-existing mating preferences against the novel colour mutation, rare mutant males mating with females with the mutant colour, will gain a frequency-dependent advantage since they will experience less intense intrasexual selection by mating with non-preferred females. This allows the build-up of an association between male mating preference for the novel colour and W-linked novel colour, so that females with the novel colouration will also carry the novel preference for the novel colour. The increase in frequency of a male rescue gene M (autosomal recessive, again, in analogy with the *N. omnicaeruleus* rescue genes), overriding the effects of the dominant female determiner, will be favoured as it creates W males with the novel colour, preferred by females with novel colour. Once a critical frequency of M is reached, complete association between W-linked novel colour, M and novel preferences for the novel colour, is rapidly generated. A new species (W'W', W'Y), fully isolated from the ancestral one (WY, YY) lacking the novel colour and the novel preference, has arisen. In conclusion, when YY are fit, Lande et al.'s (2001) simulations predict first invasion of a dominant female determiner driving reversal of heterogamety. This is followed by invasion of a marker trait linked to the female determiner and the evolution of male mating preferences for such marker trait. Eventually, a new species with the marker trait and mutual mating preferences for it is produced and heterogamety is re-established.

It is clear from this that an additional assumption of the model is the pleiotropic expression of mating preferences for the marker trait between the sexes. Mutant males with mating preferences for novel trait in females will transfer the preference to both sons and daughters. Males possessing the novel trait will be chosen by these females with the novel preference. This is indispensable for the increase of the male rescue gene M in the population.

When YY suffer reduced fitness, the intrinsic advantage of W allows its invasion. However, heavily female biased broods will be produced and eventually sex ratio selection will counteract the spread of W. As in the first model, described above, a novel marker trait is introduced at low frequencies on the W, where it is not selected against by female choice. Pre-existing male mating preferences against the novel trait create a frequency-dependent advantage for rare males choosing females with the novel W-linked colour. Individuals carrying the W-linked trait and the rescue gene will be favoured not only because females with the novel trait have a preference for the trait, but also because they produce more even progeny sex ratios than ancestral males. This again allows the increase of M in the population and eventually complete association of W-linked trait, male rescue gene M and mating preferences for the novel trait. Sympatric speciation here leads to the coexistence of one ancestral species with male heterogamety (XX/XY), absence of W and novel trait, and pre-existing male mating preferences against the novel trait, and a new species with male heterogamety (W'W'/W'Y), W-linked novel trait (i.e. W') and mutual mating preferences for the trait.

Lande et al.'s (2001) analysis might provide a key to different patterns of speciation in related groups of cichlids. Their two models differ essentially in the assumptions on YY viability. In large populations, YY individuals might, as noticed above, have similar fitness to XY. In the first model with YY viable, the invasion of W can lead to full reversal of heterogamety, this depending on the size of the YY fitness disadvantage. This in turn relaxes the constraint on recombination on the sex chromosome, making the second phase leading to speciation, less likely. Species with typically large population sizes, such as the tilapias of the genus *Oreochromis* and offshore haplochromines, are probably more prone to speciation by reversal of the heterogametic sex in populations with unbiased sex ratios. Typically, sister species in

Oreochromis are allopatric and differ in the heterogametic sex (Hammermann & Avtalion, 1979; Trewavas, 1983), as predicted by Lande et al.'s (2001) modelling.

On the contrary, rock-dwelling haplochromines are generally stenotopic and are characterised by small population sizes. Under this scenario, YY individuals are likely to suffer from the fixation of mildly deleterious recessive mutations. Lande et al.'s (2001) simulations predict an interaction between sex ratio selection, generated by the invasion of a female determiner distorting offspring sex ratios, and sexual selection on a marker trait of the distorter. Speciation is rapid, sympatric between colour morphs and does not lead to reversals of heterogamety. Sister species of rock-dwelling haplochromines frequently live in sympatry, have same heterogametic sex but differ in male and/or female colouration. Moreover, this route of sympatric divergence does not exhaust the potential for further speciation, since new sex determiners can invade and marker traits labelling them trigger the evolution of male mating preferences, eventually repeating the speciation dynamics modelled by Lande et al. (2001). Their models might then describe an "engine of speciation" that might contribute to explain the astonishing species diversity of rock-dwelling haplochromine cichlids in Lakes Victoria and Malawi.

Neochromis omnicaeruleus, therefore, might be following the pathway outlined in the second model, with YY inviable, but might be stuck in an early stage of divergence because of selection against blotched males which in turn prevents female preferences for blotch to evolve. Therefore, the issue of YY viability in this species becomes of great interest. Although Seehausen et al. (1999) and Lande et al. (2001) assume YY inviability for the rock-dwelling haplochromine *N. omnicaeruleus*, on the basis of the considerations exposed above, some of Seehausen et al.'s (1999) data might suggest the opposite, i.e. that YY individuals are indeed viable. In their breeding experiment, crosses between WB females of phenotype (c) and OB males of phenotype (c) resulted in clutches with phenotypes and sexes incompatible with the genetic model proposed. It is immediate that, since the authors found both WB and OB males in their clutches, a Y had to be present in both parents as only one W (a W_A in the mother, a W_B in the father) can be in any parent (otherwise the mother would have been W_AW_B exhibiting a WB-OB phenotype). As a consequence, a (c) morph type Y-carrier phenotype for the mother is not possible as such individual would be a male ($W_AYM_AM_A$). Although, of course, we must consider the possibility of recombination between colour and sex, an argument the authors use elsewhere (but not for this cross) to explain the appearance of rare unexpected phenotypes, a more parsimonious solution is to assume that Seehausen and colleagues incorrectly identified the colour phenotype of the blotched mother (a (c) in place of a (b) colour type). The only parental phenotypes consistent with their results are WB(b) mother (WYM_Am) and OB(c) father ($W_BYM_BM_B$). The important aspect here is that, assuming this reconstruction is correct, the plain individuals found by the authors must have been YY individuals. Although these results suggest that YY individuals are indeed viable and fit (Seehausen did not observe any evidence of loss of fitness of such P males, *pers. comm.*), more data is required to draw general conclusions.

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CHAPTER II

INDIVIDUAL MALE MATING PREFERENCE VARIATION IN A COLOUR POLYMORPHIC CICHLID FISH SPECIES FROM LAKE VICTORIA

ABSTRACT

Sexual selection theory largely rests on the assumption that populations contain individual variation in mating preferences and that individuals are consistent in their preferences. However, there are few empirical studies of within-population variation and even fewer have examined individual male mating preferences. Here we studied a colour polymorphic population of the Lake Victoria cichlid fish *Neochromis omnicaeruleus*, a species in which colour morphs are associated with different sex determining factors. We assessed within-individual consistency and between-individual variation in male mating preferences for the three female colour morphs by testing wild-caught males in three-way choice trials with multiple combinations of different females. Compositional log-ratio techniques were applied to analyse individual male mating preferences. Large individual variation in consistency, strength and direction of male mating preferences for female colour morphs was found and hierarchical clustering of the compositional data revealed the presence of four distinct preference groups corresponding to the three colour morphs in addition to a no-preference class. The repeatability of mating preferences was relatively high, suggesting a potential for evolutionary response to selection on preferences. Large individual variation in mating preferences is a pre-requisite for sympatric speciation to occur and is consistent with previous suggestions that *N. omnicaeruleus* might represent an incipient species complex. Only few males preferred the most common morph in the population. We propose that the observed preference bias against the most common female morph might be due to frequency-dependent sexual selection. Alternatively, fine-scale spatial patchiness and/or environmental fluctuations might locally favour one colour morph over the others and determine partial local segregation of male preference morphs, without affecting relative morph frequencies as well as morph preference frequencies at the population level.

Keywords

male preference, polymorphism, sexual selection, mate choice, *Neochromis*, Lake Victoria, cichlid, compositional data, log-ratio analysis, sex ratio, sex determination.

INTRODUCTION

A growing number of studies has recently documented within-population variation in female mating preferences influenced by condition (Bleay & Sinervo 2007), age (Uetz & Norton 2007), social context (Alonzo & Sinervo 2001), or targeting complex or multiple male traits (Brooks & Endler 2001; Coleman et al. 2004).

Not only is individual variation a necessary precondition for selection to act on preferences but the evolutionary consequences of such variation can be profound and contribute to determine the intensity of selection on sexually selected characters, the potential for genetic correlations to arise between traits, the maintenance of stable polymorphisms as well as the generation of disruptive selection (Widemo & Sæther 1999).

While empirical evidence for within-population variation in female mating preferences is slowly accumulating, studies documenting male choice on female signal traits are far less common (Amundsen et al. 1997; McMillan et al. 1997; Amundsen & Forsgren 2001; Bonduriansky 2001; LeBas et al. 2003; Pierotti & Seehausen 2006; Chenoweth et al. 2007) and very few have so far reported within-population variation in male mating preferences (Ryan & Altmann 2001; Roulin 1999; Pryke & Griffith 2007). Finally, only one study has examined male mating preference variation of individual males (Pierotti et al. 2008).

Male choice is expected to evolve under more restrictive conditions than female choice (Clutton-Brock & Vincent 1991; Johnstone et al. 1996; Parker & Simmons 1996). Scenarios known to be particularly favourable for the evolution of male choice include highly female-biased operational sex ratios, elevated costs of reproduction (e.g. mate search, predation risk during courtship, sperm depletion, paternal care) and/or large variation in female quality

(Kvarnemo & Simmons 1999; Kokko & Monaghan 2001; Kokko & Johnstone 2002; Clutton-Brock 2007).

A second issue is whether the operation of sexual selection by male choice on female traits in non-sex role reversed species allows the evolution of individual variation in male preferences. Servedio & Lande (2006) showed that male choice for female arbitrary traits is unlikely to evolve but a male preference for reliable signals of fecundity can be maintained in a population. Variance in female quality (here, fecundity) *per se* does not necessary lead to individual variation in mating preferences but, if females exhibit multiple genetically independent ornaments signalling different aspects of female quality, individual variation in male mating preferences might evolve. However, a perhaps more likely origin of variation in male mating preferences is sexual selection for genetic compatibility (Zeh & Zeh 1996, 1997; Tregenza & Wedell 2000). If mating preferences depend on the genotypic background of both partners, variance in mate quality for each choosing individual can potentially be very high, when the fitness of the offspring from un-matching parental genotypes is substantially reduced (Mays & Hill 2004; Puurtinen et al. 2005). Therefore, variation in male and female mating preferences might be frequently found when sexual selection is acting on genotype matching, i.e. when it is correlational (Ryan & Altmann 2001). However, in the absence of phenotypic traits revealing genotypic identity, mating preference variation does not evolve and multiple mating might ensure compatibility in at least a proportion of the clutches (Zeh & Zeh 1996). Finally, male mating preference variation can simply be the result of pleiotropy on trait and preferences between the sexes (Lande 1980).

The involvement of sexual selection in the explosive adaptive radiations of haplochromine cichlid fishes in East African lakes has been long suggested (Kosswig 1947; Dominey 1984;

Seehausen 2000; Kocher 2004) given the extreme variation in nuptial colours of closely related species and the presence of colour polymorphisms within species and frequently within populations (Greenwood 1974; Seehausen 1996; Konings 2001).

Seehausen et al. (1999) studied a population of the Lake Victoria cichlid species *Neochromis omnicaeruleus*, polymorphic for heterogamety and for male and female colour patterns. A dominant male determining factor (y), a recessive female (x) and at least two dominant female determining factors (w_A, w_B) were found in a single population in which three colour morphs coexist. The authors showed that colour genes are in linkage with the sex determiners and hence that colour is a good indicator of the sex factors carried by an individual. Evidence for male discrimination between females of different colour morphs came from two-way choice tests on fish bred in the laboratory and high variance in clutch sex ratios was observed between different parental genotypes.

Seehausen et al. (1999) proposed that sex ratio selection deriving from within-population variation in heterogamety might drive the evolution of mating preferences against sex ratio-biasing genotypic combinations (Orzack et al. 1980; Bull 1983; Wilkinson et al. 1998, Lande & Wilkinson 1999). Colour morph type in *N. omnicaeruleus* might reliably indicate a particular sex determiner as long as the latter is in tight association with colour genes. Selection for 1:1 offspring sex ratios in the population might then favour the evolution of mating preferences for genotypic combinations generating even offspring sex ratios. If such a form of genotype-matching male choice is present in a colour polymorphic species, we expect within-population variation in male mating preferences for female colour morph type.

Here we test whether wild-caught individual males, from a population of *N. omnicaeruleus* harbouring all three colour morphs, exhibit male mating preferences for female colour morph type and whether distinct male mating preference types can be identified among individual males in the population. We assess repeatability of male mating preferences across all males and within preference types as defined by the clustering.

MATERIALS AND METHODS

Study species and genetic basis of colour polymorphism

Neochromis omnicaeruleus belongs to the rapid adaptive radiation of Lake Victoria cichlids. The species is a rock-dwelling algae scraper with a lake-wide distribution on offshore rocky islands, possibly representing the most polymorphic species known from the Lake Victoria radiation. Seehausen et al. (1999) found nine female and four male distinct phenotypes in the Makobe Island population (Speke Gulf, Southern Lake Victoria). By far the most common types in both sexes are: i. a plain type (P) with dark vertical bars on a yellow-brown background in females and on a bright metallic blue background in males; ii. an orange-blotched type (OB) with large black blotches on an orange background in both sexes; iii. a piebald type (WB) with large black blotches on a white background in both sexes. Other types an be explained as hybrids and recombinants and are much less common. OB and WB males are extremely rare (Seehausen et al. 1999; Maan et al. in press). Both OB and WB blotched colouration is determined by distinct colour genes each in linkage with distinct X-linked female determining genes dominant over the male determining function of Y. As a result, blotch individuals are most often females: rare blotched males are generated by the action of autosomal suppressors specific for either OB-linked or WB-linked female determining genes. These male rescue modifiers re-establish Y male determining action so that individuals homozygote for the modifier and hemizygous for its corresponding blotch coloration and sex type are blotched (WB or OB) males. Plain males (P) lacking autosomal modifiers (male rescue factors) generate female-biased clutches when mating with blotched females which are carriers of dominant female determiners. Plain males carrying autosomal modifiers obtain more even clutch sex ratios as the action of dominant female determiners in some offspring will be suppressed by the parents' male rescue genes.

In two-way choice lab experiments, Seehausen et al. (1999) found that P females have strong mating preferences for P males, only, whereas WB females are not significantly discriminating between P and WB male colour morphs. OB females were not tested. Moreover, the authors tested plain males from plain mothers, plain males from WB blotched mothers as well as WB blotched males: such two-way male choice experiments were suggestive of mating by mother's colouration. Preliminary evidence in this species suggests male mating preferences have a genetic basis (K. Pyle and O. Seehausen, *unpublished data*) and work on a Lake Malawi cichlid species with a very similar sex-linked colour polymorphism found patterns consistent with genetic inheritance of male mating preferences (Pierotti et al. 2008).

Subjects and housing conditions

Wild males from the same polymorphic population (Makobe Island, Western Speke Gulf, Lake Victoria) previously studied by Seehausen et al. (1999) were collected in the year 2003, scuba diving by hook and line at a depth of 0-3m. Of these, sixteen belonged to the P morph (males 1-3, 5-17), three were of OB morph (males 18-20) and two of WB morph (males 21-22). Our experimental sample also included two wild P males from a collection made in 2001 (4 and 25) and two lab bred F1 WB males (23-24), deriving from WBxWB crosses of the 2001 collection.

Finally, one lab bred male from a line developed from PxP crosses of an earlier collection was included (male 26).

Prior and during the period of the experiments, males were isolated in individual compartments with no visual contact with any other fish. Females were kept inside large tanks in transparent plastic enclosures provided with large holes ensuring water flow. The same type of transparent plastic jars was used to confine females in the experimental tanks during male preference trials. All aquaria were maintained at 26 ±2°C and illuminated with daylight fluorescent bulbs on a 12:12h light:dark cycle. Fish were fed twice a day with flake food and a vitamin-enriched mix of mashed prawns and peas.

Mate preference trials

Each male was tested with a different set of 6-11 combinations of females, every combination consisting of a female for each colour morph (P, WB, OB). No male was tested more than once with the same female and same female combination. No female combination was used more than once in the entire experiment. Females within each combination were size-matched ($\leq 5\%$ SL difference). There was no difference between female morphs with regards to standard length, weight or gravidity stage (Friedman tests, $P > 0.05$).

Three transparent plastic enclosures were placed at equal distances on the back side of a 2720-litre experimental aquarium. Females were placed in the experimental tank before the introduction of a test male. A trial started with the first encounter, defined as a male approaching a female to a distance less than 20 cm. Trials in which the male failed to approach all females at least once were discarded.

Courtship and aggressive behaviour of test males were scored continuously for a period of 15 minutes per trial, following Baerends & Baerends van Roon (1950).

Statistical Analysis

We measured male mating preferences as the proportion of male courtship displays towards each of the three female morphs. Hence, an individual's preference in each trial is described by three ratios (the proportions of displays to each female morph), i.e. by three components with unit-sum constraint. For each trial, we are interested in the relative ranking of female colour morphs generated by the experimental male and not in the total number of courtship displays in a trial. However proportions (and in general, ratios) are not suitable for standard multivariate analysis (e.g. there is no exact relationship between the variance of x_i/x_j and x_j/x_i; see Aitchison 1986 for a review of the problems associated with analysis of ratios). For this reason, we applied logarithmic transformation of the ratio data, according to the methods of compositional data analysis (Aitchison 1986; 1999). In particular, we performed an isometric log-ratio transformation (ilr) (Egozcue et al. 2003) to the proportion of male courtship displays towards each female morph per individual male (see Appendix A for definitions and details).

Before log transformation of the dataset was possible, observations with zero values, i.e. trials containing zero courtship displays towards one of the stimulus females, had to be modelled with appropriate compositional techniques (Aitchison 1986; Egozcue et al. 2003; Martín-Fernândez et al. 2003) in order to preserve the true total number of displays per trial and the ratios between the variables.

Each individual male can be imagined as represented by three components corresponding to its mating preference towards each female colour morph (P, WB, OB). These three components have unit-sum constraint and identify a preference vector characteristic of that individual male, a *compositional geometric mean vector* of preferences for that male (Aitchison 1986). All males, characterized by their compositional geometric mean preference vector, were plotted on a ternary diagram (Fig. 1A) to identify patterns in the distribution of individual male preferences for female colour morph. In Fig. 1B, confidence regions calculated under log-ratio normality assumption are added to plots of each individual male and the overall dataset.

A MANOVA was applied in order to analyze the differences between the geometric mean of individual male mating preferences. Equality of the covariance matrices for the 26 males was confirmed with a Box's M test.

We explored the distribution of preference groups corresponding to the three female colour morphs using conventional hierarchical clustering. Geometric means of individual males were clustered using log-ratio Mahalanobis distance (Aitchison 1986) following the average linkage method. By employing Mahalanobis distances, we make sure that the amount of dispersion exhibited in the single preference trials belonging to a certain male (the preference vectors for that male) is taken in account in the calculation of distances between males. In other words, not only the mean preference but also both the strength and the consistency of mating preference of an individual male influence its distance from other individuals. In fact, the Mahalanobis metric is a weighted Euclidean distance where the weighting is determined by the range of variability of the sample point as expressed by the covariance matrix.

Therefore, the variance and the correlations of the variables are accounted for in the distance between one male and a second male, i.e. the *distribution* of the preference vectors influences the distance value between any two males.

In the clustering procedure, when two groups were merged, the pooled covariance matrix was assigned to the resulting group and was used for calculating the new distances between individuals.

The results of the cluster analysis were used to derive a dendrogram and plotted on the ternary diagram representing the distribution of the mean compositional mating preferences of individual males (Fig.2A, B). A series of complementary techniques and indices (Jobson 1992) was used to assess the quality and stability of the clustering results. (see Appendix B).

Repeatability (Lessells & Boag 1987; Boake 1989) both across the total male sample and across the preference groups (as defined by the clustering procedure) were calculated. We developed multivariate versions of repeatability using two different strategies (see Appendix C) either replacing the univariate variances in the repeatability formula with the corresponding trace of the covariance matrix or applying the relationship between repeatability and F ratio for the analysis of variance from an F statistic (Lessels and Boag 1987; Jobson 1992). Repeatability measures were then studied by Monte Carlo-based bootstrap simulations of the original compositional dataset.

All calculations were performed with the Statistics Toolbox 6.1 of Matlab© 7.01 (2005).

RESULTS

Individual male mating preferences for female colour morphs were measured on 26 *N. omnicaeruleus* males, over 205 successful trials. We found large individual variation in male mating preferences as evident from the distribution of the geometric means for each male plotted in the ternary diagram (fig. 1A,B).

The existence of differences in the geometric mean of individual male preferences is also supported by the MANOVA analysis (p-value<0.001) applied to the log-ratio transformed dataset. Homogeneity of log-ratio variances and covariances was confirmed by the Box's M test (p-value = 0.0603). This equality of covariances is a hypothesis for the MANOVA and it is implicitly assumed by the clustering procedure with the log-ratio Mahalanobis distance.

Some individual males exhibited extremely strong mating preferences for a certain female colour morph (e.g. males 9, 10, 12, 13, 23, 25) as can be seen from the location of the mean close to one apex of the ternary preference space and the area of the confidence region around it in fig.1B.

Clustering of individual male mating preferences identified three completely distinct groups of males with individual preferences for WB, for OB, for P females, and a fourth group with no preference bias towards any colour morph (Fig. 2A,B). The geometric means of individual male preferences for each female colour morph in the 4 classes of male preference are also shown in Fig. 4.

While ternary diagrams provide a useful tool to describe compositional data such as relative preferences towards multiple stimuli, they do not represent data in a Euclidean space and therefore prevent a quick 'bird's eye' assessment of relative distances between individuals. Log-ratio plots, though less intuitive than the ternary, represent distances in a usual Euclidean space and can provide a better view of the relative dispersion in space of the geometric means of individual male preferences. The 3 log-ratio plots of the zero-adjusted courtship displays are shown in Fig.3. The apparent linear relationship between log(P/WB) and log(OB/WB) suggests that the majority of males vary in their preference for WB females, from preference *against* to strong preference *for* females of that morph but they give a similar assessment of the breeding value of P and OB females, sharing a common ratio of preferences for P and OB females.

Repeatability estimates obtained after bootstrap Monte Carlo simulations on the compositional dataset are summarised in Tab. 1. Repeatabilities calculated from the traces of the variance matrix were systematically higher than when calculated from F statistic.

All wild-caught individuals from the 2003 field collection and the blotched first generation lab-bred individuals from the 2001 collection did not occupy a large portion of compositional space, and in particular the regions of preference for P females (yellow areas in the ternary plot) as well as the region characterising a OB > P > WB hierarchy of male preferences. In other words, the preference class of males ranking P females as superior to OB ones was absent from the wild caught 2003 sample as well as from both first generation lab-bred WB males from the 2001 sample. However, the lab-bred individual (male 26) and, to a less extent, the wild P male from the 2001 sample (male 25), showed mating preferences for P females.

While wild-caught OB males and lab-bred WB males showed mating preferences for females of their own colour morph, as suggested for OB and WB males by Seehausen et al. (1999), in our experiment WB wild-caught males exhibited preferences against WB females (Fig. 1).

DISCUSSION

Our study provides evidence for dramatic individual variation in male mating preferences for female morph types in the colour polymorphic Lake Victoria cichlid *Neochromis omnicaeruleus*. We found large variation in both strength and direction of male mating preferences and a compositional hierarchical clustering identified highly distinct preference classes corresponding to regions of strong preference for the three female colour morphs as well as a group of non-discriminating individuals. This 'no-preference' class is not formed by an artificial grouping of males with very different mating preferences but, on the contrary, represents a homogeneous group, as shown by the dispersion of the trials data around the geometric means of each individual.

With some limitations (Dohm 2002), repeatabilities can provide an upper limit to heritability and lend important insights in the potential for selection to act on the repeatable trait. Male mating preference repeatabilities across all males were moderately high and with low standard deviation, in comparison to published estimates of female mating preference repeatability (Boake 1989; Bakker & Pomiankowski 1995; Wagner et al. 1995; Brooks & Endler 2001; Cummings & Mollaghan 2006). Although it is the additive genetic component what really matters for the potential for evolution of a trait and not simply its heritability, the repeatability values of male mating preferences for female colour morph patterns in our study suggest that there might be sufficient heritable variation for preferences to rapidly evolve.

The large individual variation in male mating preferences for alternative colour morphs might generate conditions for disruptive sexual selection. Van Doorn (2004) showed that when a polymorphism in male trait/female preference is stabilised by negative frequency-dependent selection, disruptive sexual selection on male colour pattern can drive speciation. It is unclear whether a polymorphism in female trait and male mating preferences might drive similar macroevolutionary dynamics.

When variation in female colouration and sex determining factors become associated, linkage alone might initiate divergence of male mating preferences between colour morphs and eventually lead to sympatric speciation (Lande et al. 2001). In *N. omnicaeruleus*, females carrying sex ratio distorting genes (e.g. dominant female determiners invading male heterogametic systems) are 'labelled' by linkage disequilibrium between the distorting genes and colour markers, the OB and WB blotch genes. Some males mating with blotched females suffer heavily female biased clutches. However other males are immune from these effects when mated with blotched females. These males carry autosomal modifiers that over-ride the effects of the sex biasing genes carried by blotched females. Seehausen et al. (1999) proposed that males with preferences for non-blotched females lack the autosomal modifiers whereas males with preferences for blotched females are carriers of the modifiers. The large variation in individual mating preferences for female colour markers of sex ratio distorting genes (i.e. blotched colouration OB, WB)found in our study provides support to Seehausen et al.'s (1999) hypothesis. However it remains to be shown whether the experimental males exhibiting preferences for OB or WB blotched females in our study are also carrying autosomal modifiers while males with preferences for P females lack autosomal modifiers, as suggested by Seehausen et al. (1999). Crosses making use of our wild male sample are now underway to test this proposed correlation between male mating preferences and autosomal modifiers of sex ratio distortion.

Selection for compatible mates between colour morphs (e.g. generating unbiased offspring sex ratios) might generate correlational selection creating favourable genetic associations between suites of traits, i.e. coadapted gene complexes (Brodie 1992). Non-random mating might then allow genetic correlations not supported by pleiotropy but deriving from linkage disequilibrium to be maintained in the face of recombination. The interplay of correlational selection and assortative mating might then lead to population divergence in sympatry (Lande et al. 2001; Sinervo & Svensson 2002).

We do not know whether the alternative mating strategies of individual males towards female morphs emerging from our study are driven by sex ratio selection caused by coexistence of x, y and w sex determining factors with w factors in linkage with colour genes or underlie other fitness effects associated by linkage or pleiotropy to OB and WB colouration. In any case, the large variation among males in strength, direction and consistency of male mating preferences for female morph type is consistent with compatibility-driven mate choice.

However, a negative frequency-dependent advantage in male-male competition over females of different morph types can also generate an association between female morph and male mating preferences for female morph (Lande et al. 2001; Servedio and Lande 2006). There is some suggestion that this might be taking place in our population of *N. omnicaeruleus*. In fact, a rather unexpected result of our study was the lack of males with preferences for plain females as evident from the large empty portion of the ternary plot (fig. 2). The experimental design did allow detecting male mating preferences for P females: males 25 and 26 exhibit a preference bias towards P. However these two males are not belonging to the main experimental sample from the 2003 field collection: male 25 being a wild male from a sample of 2001 and male 26 a lab bred individual deriving from the 1999 collection. The frequency distribution of male mating preferences is particularly puzzling since plain females are the most common morph in the population and relative frequencies appear to remain relatively stable over ecological time (Seehausen et al. 1999; Maan et al. 2008). While the most common female morph in the population corresponds to the rarest male preference in our sample, the rarest female morph (WB) in the population corresponds to the most common preference morph (preference for WB females) in our sample, suggesting a possible "rare mate" effect in the dynamics of male mating preference frequencies. When polymorphism in preference and trait under mate choice exists in the population and there is intrasexual competition, sexual selection is necessarily frequency-dependent, potentially leading to rare mate effects (O'Donald & Majerus 1988).

The build-up of linkage disequilibrium and gene complexes through the correlational selection generated by assortative mating on male rescue genes is strictly dependent on selection for optimal sex ratio in a population in which male rescue alleles are at low to intermediate frequencies. Evidence from intraspecific competition studies (Dijkstra 2007) suggest that blotched females might have a competition advantage over non-blotched females. Nevertheless, blotched individuals might be penalised by being carriers of sex ratio distorting genes. Moreover, Seehausen et al. (1999) proposed that fixation of sex ratio rescue genes in *N. omnicaeruleus* might be hampered by higher predation on blotched males, as suggested by the experimental work of Maan and collaborators (2008). Under this scenario, if environmental conditions such as water turbidity or predation pressure were to relax selection on blotched males, male rescue genes might increase to become very common. Sex ratio distorting genes, fully compensated by the spread of male rescue modifiers, would not anymore exert sex ratio selection on mating preferences and therefore the fitness of different colour morphs would become disentangled from genotype matching. Selection for mate compatibility would then be converted into selection for 'good genes', possibly also converting non-additive genetic variance deriving from correlational selection into additive genetic variance.

However, if the unanimous male mating preference against plain females observed in this study were due to a change in the relative fitness relationships between plain and blotched females (e.g. spread of male rescue genes as a consequence of relaxation of selection against blotched males) so that blotched females had overall higher fitness than plain females, we would predict a corresponding significant change in colour morph frequencies in the wild as a direct consequence of the shift in fitness value of blotched individuals, but this was not the case (Seehausen et al. 1999; Maan et al. 2008). This signature of selection on allelic frequencies would also be expected to appear very rapidly given the specific genetic architecture of blotched colouration (i.e. blotch being dominant over plain).

Perhaps, since sexual selection on a polymorphic trait generates frequency-dependent selection (O'Donald & Majerus 1988), male mating preference variation might be maintained even when one colour morph has higher intrinsic fitness, when intra-sexual competition favours the rarer less preferred morph (Lande et al. 2001; Servedio & Lande 2006). Even low frequency of P preferring males might buffer the effects of selection and delay measurable changes in relative morph frequencies.

Previous estimates of relative morph frequencies at Makobe were obtained by sampling at various sites along the coast of this tiny island, but the 2003 experimental male sample tested in this study was collected at one site only. Although the population as a whole might be represented by the morph frequency distribution found by Seehausen et al. (1999) and by Maan and co-workers (Maan et al. 2008) and that this might remain stable over long periods, this might not be the case at a smaller scale.

N. omnicaeruleus are epilithic algae scrapers and males defend feeding and mating territories approximately centred on a hide-out or refuge. If suitable mating territories and hideouts represent a limited resource in the highly packed community of a Lake Victoria island reef, producing slightly female-biased clutches might then be selectively advantageous. Fluctuations in environmental parameters such as water turbidity, affecting light penetrance and therefore aufwuchs' abundance and distribution, might relax or intensify intra-sexual competition for territories and locally favour biased offspring sex ratios through strategic allocation of mating resources to partners carrying sex ratio distorting genes.

Variable environmental parameters might affect male mating preferences in another way. Plain and blotched females are probably differently affected by predation (Maan et al. 2008) but this might be, at least in part, depending on background matching effects. It is possible that blotched females might be concentrating in areas where the natural background provides higher crypsis by facilitating disruptive effects on silhouette while plain females might be less exposed to visual predators over more homogeneous backgrounds with a matching light spectrum. If females of one morph spend even just a higher fraction of their time on different sites from where the other female morph spends its higher fraction of time, it would be beneficial for males with strong mating preferences to establish their territories in areas where females of the preferred colour morphs are more likely to be cruising through a male's territory.

Our results might then be the result of fine-grained distribution of mating resources and sampling at a different site might have possibly resulted in plain males being over-represented in the sample. Experimental field work is needed to test this hypothesis.

Small-scale temporal fluctuations in the frequency of female morphs might also be responsible for changes in the frequency of mate preference classes as shown by Sinervo (2001) in the colour polymorphic side-blotched lizard, *Uta stansburiana*.

Finally, the apparent lack of male mating preferences for plain females, the most common female morph in the population, might be due to state-dependent switches in mating preferences as, again, observed in the side-blotched lizard (Bleay & Sinervo 2007). In this species, females not only differ in individual mating preferences for male colour morphs but modify their preference function after having laid their first clutch of eggs. This switch might be condition-dependent or being part of a strategy of mating resource allocation. Similar considerations might apply to our case. Cost of mating for a male might be higher with blotched females that are more aggressive than plain ones but just for the same reason, blotched females might represent better mothers since they might defend grazing territories and their clutch from predators and conspecifics more efficiently than plain females. It is possible that males might have condition-dependent strategies related to their perception of future opportunities for mating or mortality risk in the experimental set up. Alternatively, as shown in other species (Uetz & Norton 2007), mating preferences might change during life-time. Our experimental males were caught in the wild when fully mature and testing was performed after roughly a year since collection i.e. roughly in the second to third year of reproductive life. Limited residual reproductive opportunities might lead to drastic switches of preference functions. In both condition-dependent and life history-dependent scenarios, the patterns of choice observed suggest that blotched females are preferred: possibly males might allocate their last opportunities in more risky but more rewarding matings with blotched rather than plain females.

In conclusion, we have provided evidence for large within-population variation in male mating preferences for female colouration in a polymorphic population of a Lake Victoria cichlid species. Individual males differed in the strength, direction and consistency of their mating preference for the three female colour morphs, present in the population. This is the first time, to our knowledge, that individual variation in male mating preferences for a polymorphic female trait is uncovered but is consistent with recent models of rapid sympatric speciation (Lande et al. 2001) by sexual selection.

Finally, this is the first time, to our knowledge, that compositional data analysis is applied to sexual selection studies. We hope that this work might stimulate interest in the application of this relatively young but powerful statistical approach (Aitchison 1986; Aitchison et al. 2000) to the study of other natural polymorphisms.

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Fig. 1. A) Ternary diagram showing the geometric means of male mating preferences for 26 *N. omnicaeruleus* individuals from Makobe Island population. Each fish in the ternary diagram represents its compositional geometric mean of preferences towards P, WB and OB females. (Hence, male preference bias towards one female colour morph increases from one side (0%) of the triangle to the opposite vertex (100%)). Males 1-3 and 5-17 are P wild 2003; males 18-20 are OB wild 2003, 21-22 WB wild 2003. Males 4 and 25 are P wild 2001 and 23-24 are lab bred F1 WB sons of wild 2001 parents.

B) Ternary diagrams showing geometric mean and its confidence region of individual male mating preference for the 26 *N. omnicaeruleus* individuals from the Makobe Island population. Each point in the ternary space (simplex) represents the compositional geometric mean of male mating preferences towards P, WB and OB females.

Fig.1A

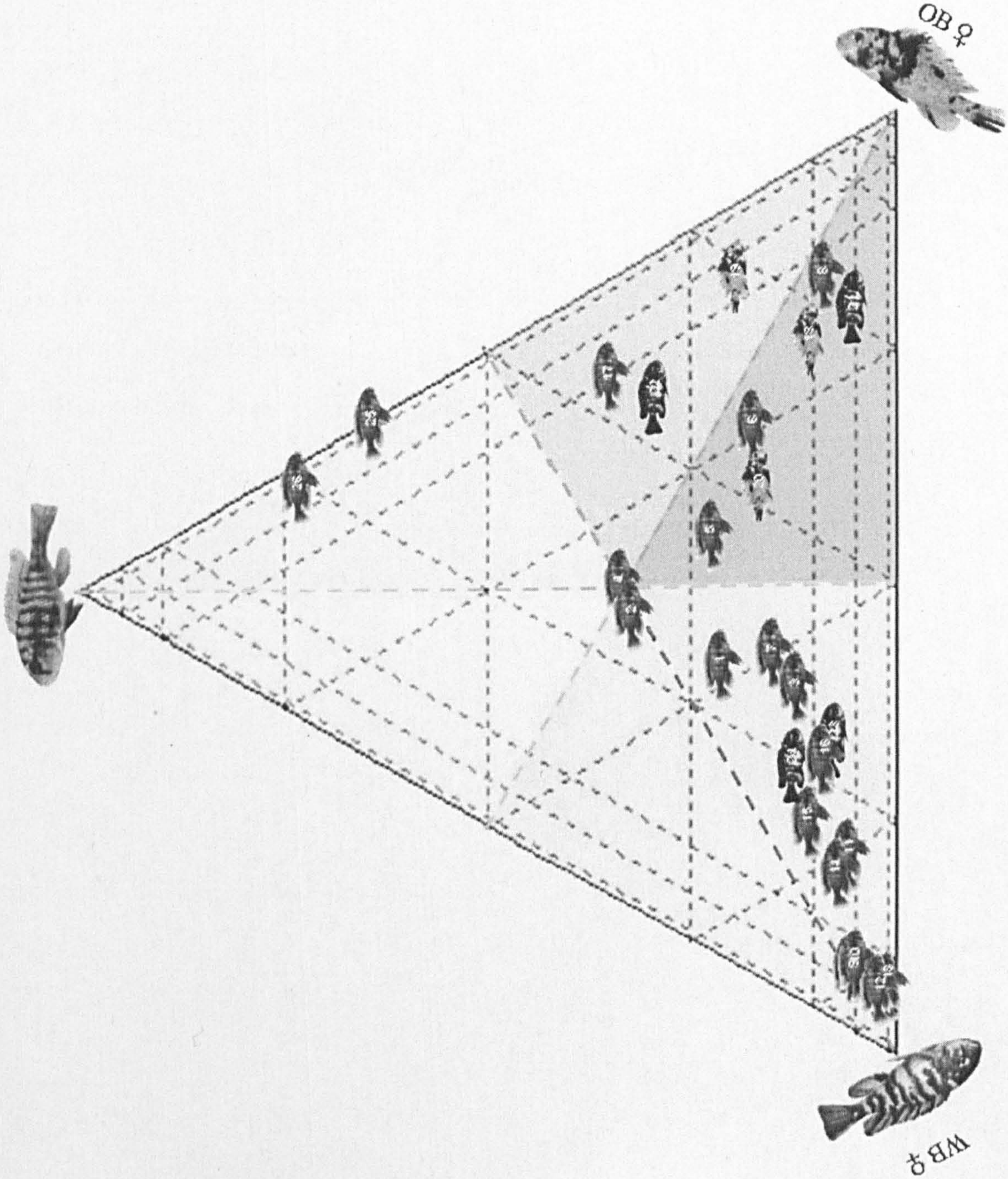


Fig. 1B

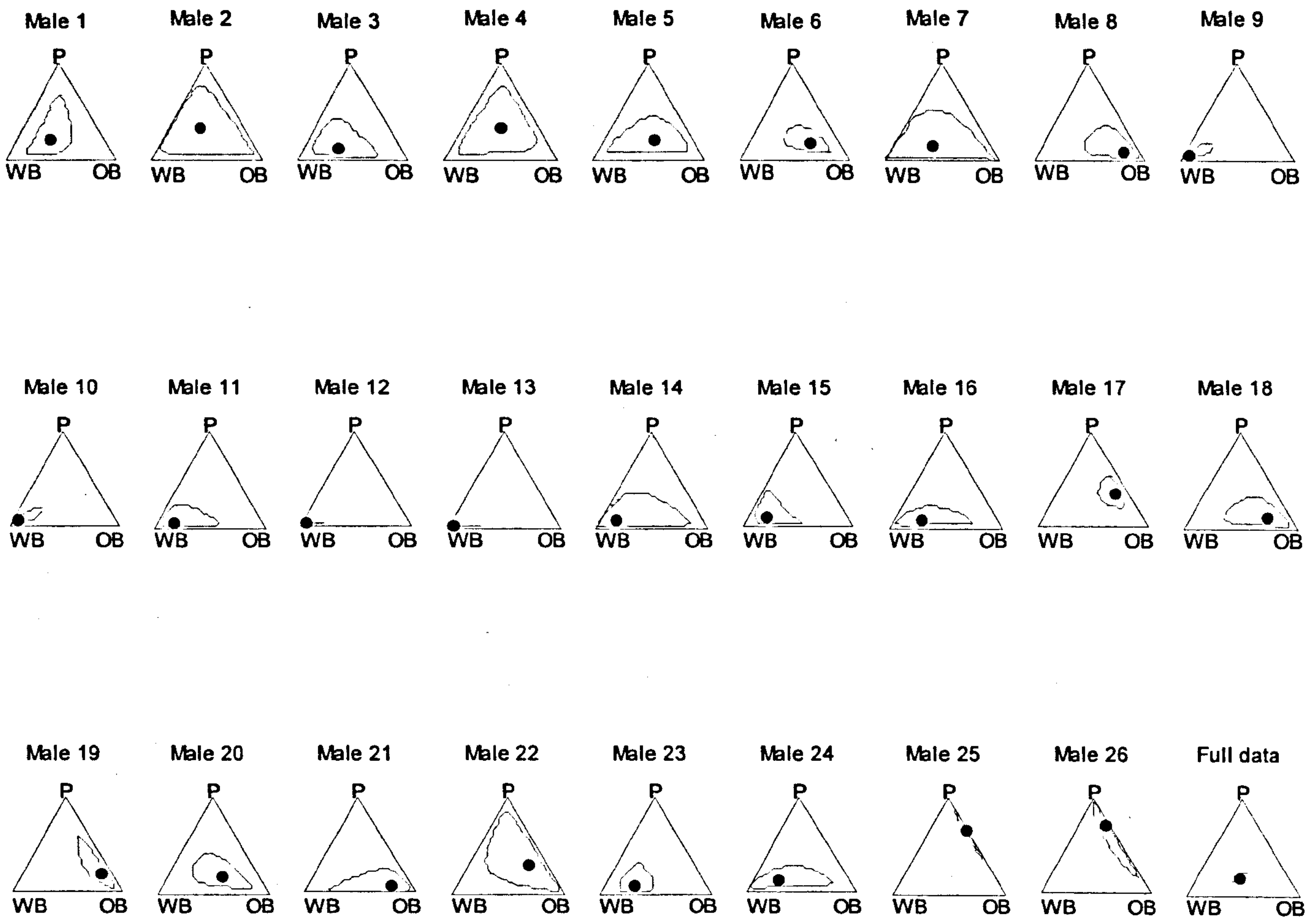
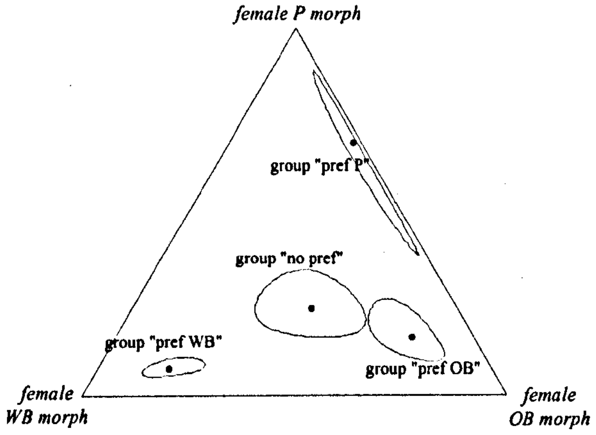
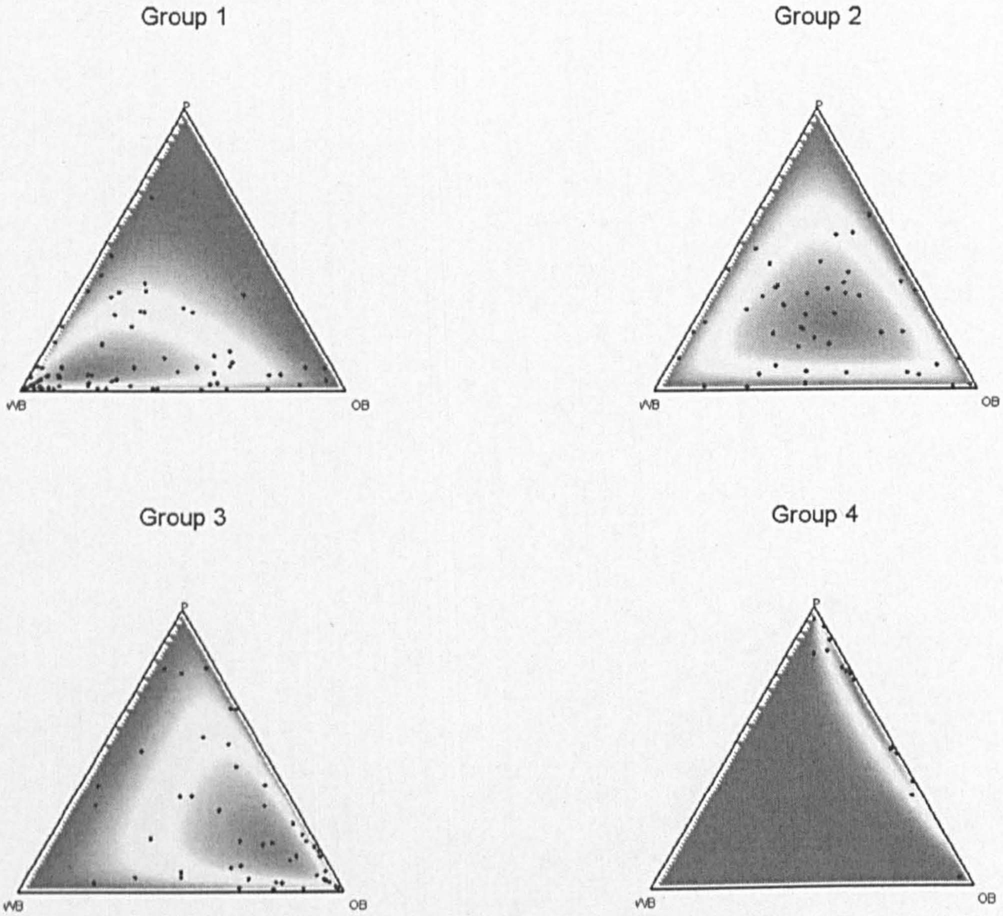

Male 1
Male 2
Male 3
Male 4
Male 5
Male 6
Male 7
Male 8
Male 9
Male 10
Male 11
Male 12
Male 13
Male 14
Male 15
Male 16
Male 17
Male 18
Male 19
Male 20
Male 21
Male 22
Male 23
Male 24
Male 25
Male 26
Full data
P
WB
OB

Figure 2. A) Male preference classes as generated by a hierarchical cluster analysis of individual male preferences for female colour morphs (see main text and Appendix B). Compositional geometric mean and confidence region for each preference class are plotted in the ternary diagram.

B) For each of the four male mating preference classes identified, individual data points and distribution surfaces are shown.



(A)



(B)

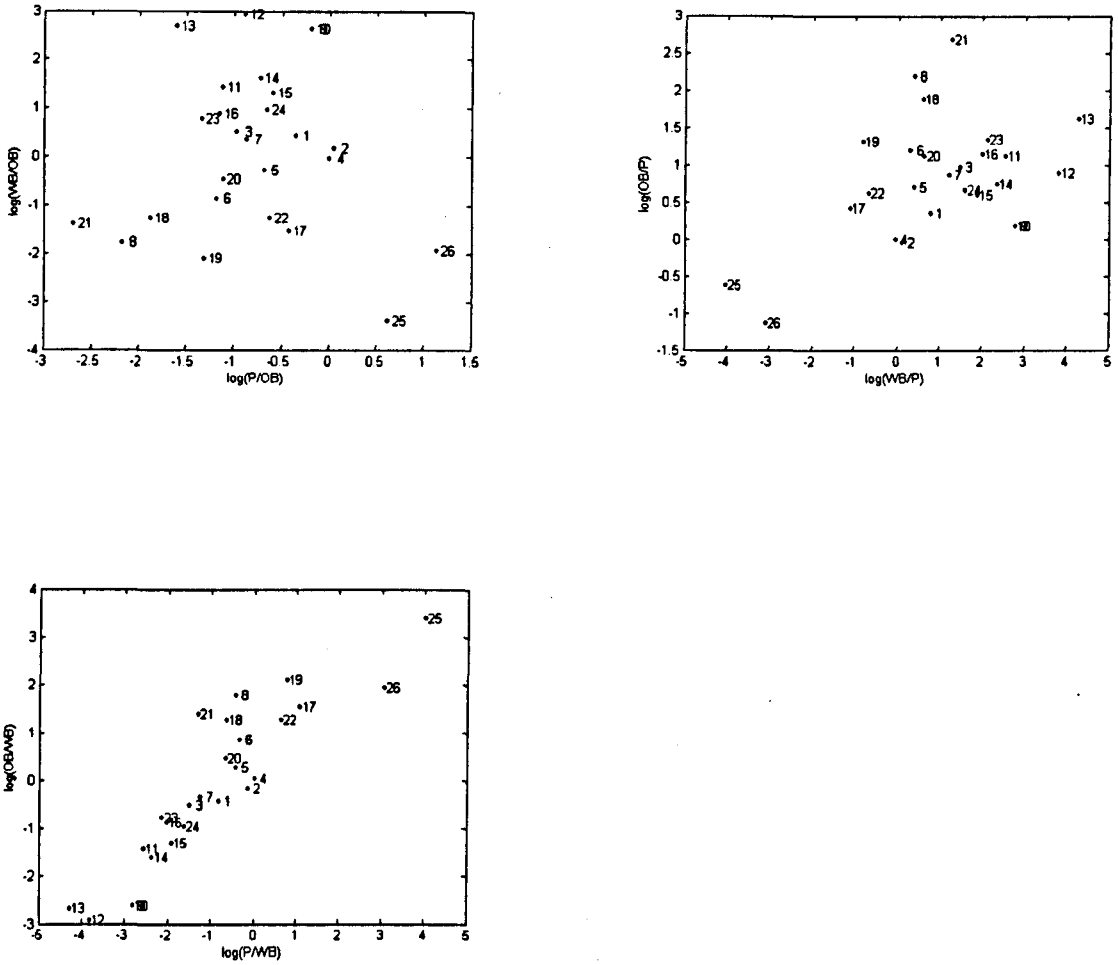


Fig. 3 Log-ratio plots (on log ratio-transformed data) of geometric means of male mating preferences. These plots allow visual exploration of real Euclidean distances between individual males, a possibility precluded in the *simplex*, the non-Euclidean space in which ternary diagrams (e.g. figs 1,2) are plotted.

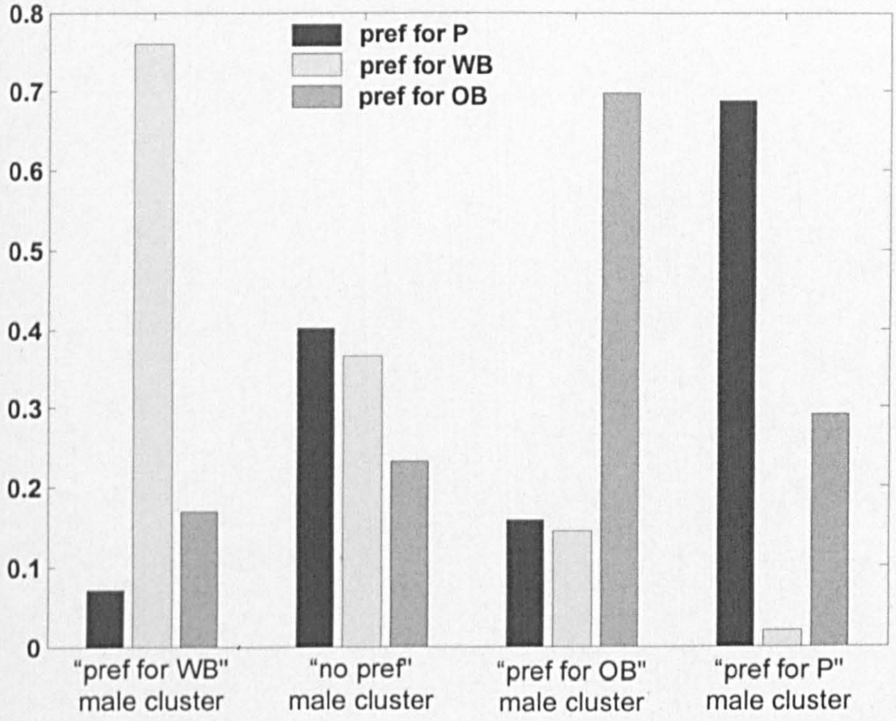


Fig. 4. Bar plot of geometric means of male mating preferences for the four male preference classes identified by the hierarchical clustering.

		Repeatability across all 26 males		Repeatability across the 4 preference groups	
		Trace	F ratio	Trace	F Ratio
	Sample	0.388	0.369	0.448	0.421
Bootstrap	Mean	0.465	0.449	0.457	0.432
	St. dev.	0.035	0.035	0.036	0.033
	$P_{2.5\%}$–$P_{97.5\%}$	0.400–0.533	0.381–0.522	0.384–0.529	0.365–0.495

Table 1. Repeatability of individual male mating preferences for female colour morphs across all experimental males and across male preference groups identified by the cluster analysis. Both the trace and the F ratio approaches for the calculation of repeatability are presented and the mean, s.d. and percentiles are provided for the estimates generated by bootstrap Monte Carlo simulations.

Appendix A: *Compositional data, ilr transformations and Bayesian-multiplicative treatment of count zeros.*

Any set of data representing proportions of some whole is said to be compositional and is subject to the constraint of summing up to 1. This can be formalized by considering the set of proportions as a vector $\mathbf{x} = [x_1,\dots,x_D]$ of non-negative elements $x_1+\dots+x_D$ representing proportions with unit-sum-constraint $x_1+\dots+x_D = 1$. Compositional datasets therefore consists of groups of such vectors of proportions. In our experiment, each vector represents a trial consisting of the three values of proportions of courtship displays towards each of the three female colour morph stimula.

The usual techniques of multivariate statistics cannot be directly applied to proportions or ratios because of the unit-sum-constraint, although there is a long history of studies ignoring the so-called *compositional problem* and gathering wrong conclusions by erroneous direct application of conventional statistical analysis to ratios (Aitchison 1996).

Appropriate log transformations provide a one-to-one correspondence between compositional vectors of proportions and log-ratio vectors in real space, allowing the use of standard multivariate techniques on the transformed dataset. Here we applied the isometric log-ratio transformation (ilr) (Egozcue et al. 2003) to the proportion of male courtship displays towards each female morph per individual male. The ilr transformation is defined by

$$\mathrm{ilr}(\mathbf{x}) = \mathbf{y} = [y_1,\dots,y_{D-1}] \in R^{D-1}, \text{ where } y_i = \frac{1}{\sqrt{i(i+1)}} \ln\left(\frac{\prod_{j=1}^{i} x_j}{(x_{i+1})^i}\right),$$

where $\mathbf{x}$ represents the vector $\mathbf{x} = [x_1,\dots,x_D]$ of proportions with D components.

When applying a log transformation of the data, the problem arises of how to deal with the log transformation of zero values in the original dataset (i.e. log 0 → ±∞). Zero values in general are of two alternative types. False or *rounded* zeroes are due to accuracy limitations of the analytical instrument of measurement, preventing the collection of small (or infinitely small) values. Martín-Fernández et al. (2003) discusses the treatment of rounded zero values in compositional analysis. Here, on the contrary, we deal with so-called *count* zeros, i.e. conditions in which a zero value might be qualitatively and not only quantitatively different from other proportion values (1/n, 2/n, ...). In particular, in our experiment, if a male does not court even once a female of a certain morph during a trial, this might not be only qualitatively different from courting her once or twice or many times in that trial, but might underscore a qualitative difference in the preference function of that male, possibly a preference against that particular female morph. As we cannot exclude this possibility, e considered zero values in our dataset as count zeros and modelled our observations with zero values by a replacement strategy following a mixed Bayesian-multiplicative approach. Our model preserves the true total number of displays per trial and the ratios between the variables. In this way, we minimize the distortion of the covariance structure for the observed part of the dataset.

After Walley (1996), we took a Dirichlet distribution as a non-informative *a priori* t ($s = 1$, $\alpha = t = 1/3$) for the replacement.

The counts vector **c** in the space [C] derives from a multinomial distribution with D categories. If N is the total count in **c** and θ is the parameter vector of probabilities, then the prior distribution for θ is the conjugate distribution of the multinomial: a Dirichlet distribution of parameter vector α, where $\alpha_k = st_k$, $k=1,\ldots, D$. We define *s* as the scalar "strength" of the priori;

and **t** as the "priori" expectation for θ. After one sample is collected, the posteriori distribution for θ is a Dirichlet distribution of parameter vector α^*, where $\alpha_k^* = c_k + st_k$. Here c_k is the observed counts in the category k of vector **c**. Therefore, Bayes theorem gives the following posterior estimation for θ_k

$$\hat{\theta}_k = \frac{c_k + st_k}{N + s}$$

where

c_k is the number of courtship displays to a specific female,

N the total number of displays in a certain trial,

t_k expected probability 'a priori'

s the *strength priori* parameter

When we assume a *priori* non-informative model (or uniform model), then the value of t_k is equal to 1/D. Therefore, in our experimental design $t_k = 1/3$. s is a parameter that controls for the effect (or weight) that the prior distribution of probability has on the posterior distribution of probability. Note that if s = 0, then the posterior is equal to c_k/N and only depends on the observed data in the trial, and the prior **t** has no effect on the posterior distribution. When s = 1 and $t_k = 1/3$, for each proportion vector **x** in [X] the replacement of the zeros is

$$x_k = 0 \text{ replaced by } r_k = \frac{1/3}{N+1},$$

that is, impute (1/3)/(N+1) in each component with null value in the vector **x**. The non-zero values (part per one) are "readjusted" in a multiplicative way using the formula

$$x_k > 0 \text{ replaced by } r_k = x_k(1 - \sum_{x_k=0} r_k).$$

In this way the ratios between non-zero components and the unit-sum constraint are preserved. After the replacement we have a new data set [XR] (part per one) without zeros. We can also make the data set [CR] (counts) without zeros if we multiply each row of [XR] by the total count of each row in [C]. This strategy has the advantage that the initial total count is preserved.

We examined the raw dataset [C] (counts of displays to each female morph per trial per male), the proportion of displays dataset [X], the zero-adjusted counts dataset [CR] and the adjusted proportions dataset [XR], by comparing their univariate distribution (Fig. 1, Appendix A) and their distribution in a ternary diagram (Fig. 2, Appendix A). The distribution of individual data points suggests no significant differences between the datasets with zeros (C, X) and without zeros (CR, XR). Minimum and maximum observed values in the CR and XR datasets confirmed that there are no notable deviations in the data distribution and values after zero replacement.

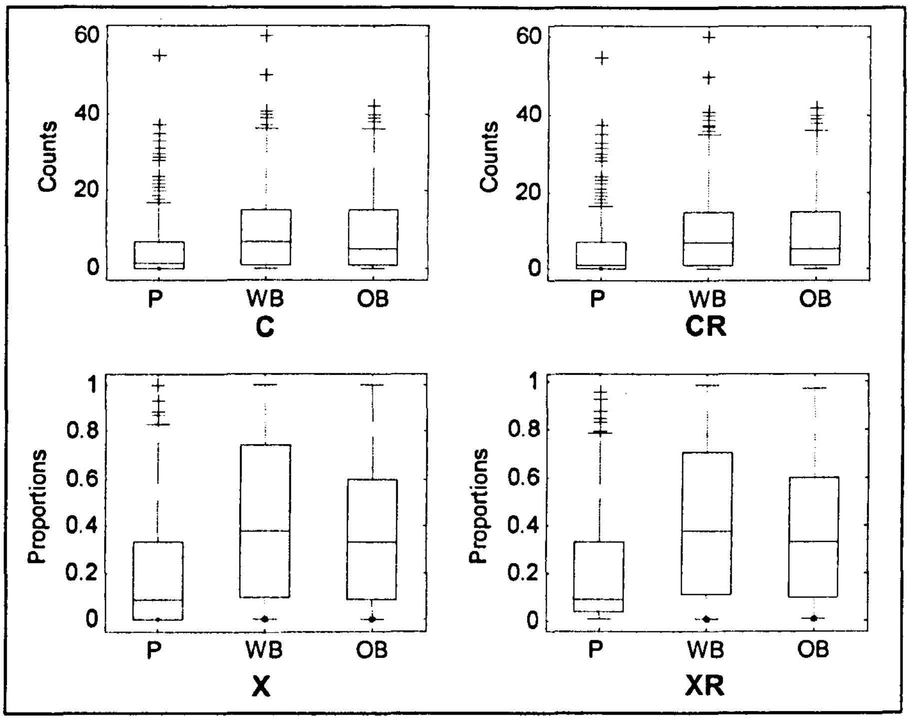


Fig.1 Appendix A. Comparing distributions of raw and transformed data: Box plots of the raw dataset [C] (counts of displays to each female morph per trial per male), of the zero-adjusted counts dataset [CR], of the proportion of displays dataset [X] and the adjusted proportions dataset [XR].

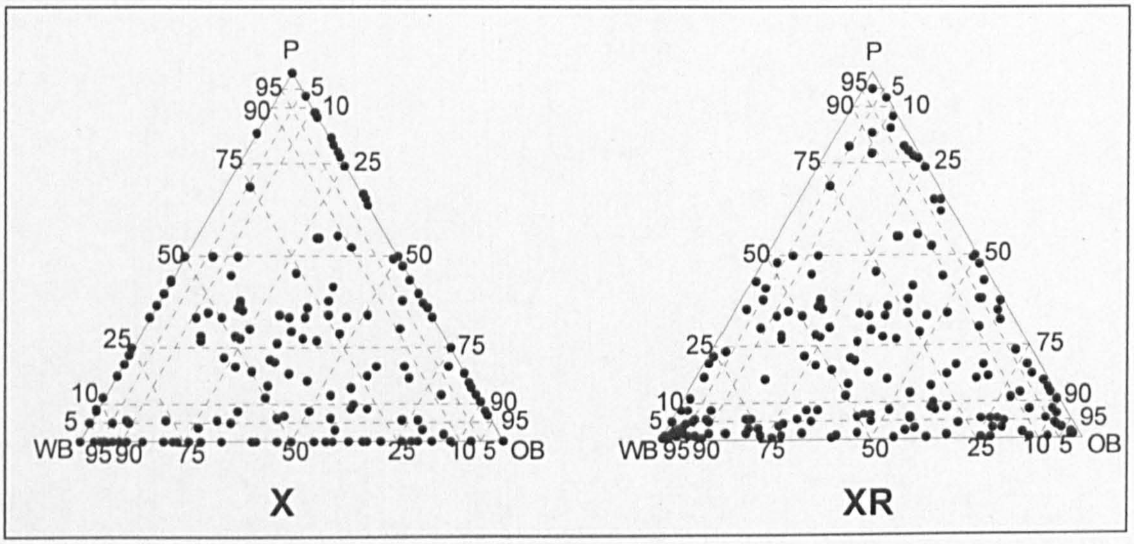


Fig.2 Appendix A. Comparing distributions of raw and transformed datasets on ternary diagrams. Symbols as in Fig. 1.

Appendix B: *Clustering validation*

We used two complementary approaches for cluster validation:

1. *Stability* was assessed by exploring the behaviour of different hierarchical methods applied to the geometric means of males using Mahalanobis distance and the biological relevance of differences in the results between methods examined.

2. In order to estimate the *quality* of the clustering, we used the *cophenetic correlation* and the *stress index* and applied them to all five hierarchical methods considered. The "true" number of clusters was assessed by Mojena index (Mojena, 1977), gamma index and point biserial. Finally, we calculated the Calinski & Harabasz's index (Calinski & Harabasz 1974) which generates a ratio between the trace of the between- and the trace of the within-covariance matrixes, with large values indicating well separated and homogeneous clusters. All these indices for different number of clusters were plotted for each clustering algorithm considered and the pattern of variation among them compared.

The stability of the cluster analysis and its performance indexes as well as the repeatability measures were studied in a bootstrap simulation based on a Monte Carlo process. This procedure of resampling consisted on simulated 1000 random samples from the full data set. In each round, the randomly generated sample preserved the existence of trials by males or groups resulting from the cluster analysis. For each random sample, the corresponding indices were calculated and then mean, standard deviation and 97.5% and 2.5% percentiles for the 1000 samples were calculated (Table 2 in Appendix B).

Results of bootstrap simulations for repeatability estimates are provided in Tab. 1, main text. Algorithms based on complete linkage, average linkage, centroid and Ward methods produced similar dendrograms, suggesting stability of the clustering. Ambiguity between methods was limited to the assignment by the centroid and the average methods of three individuals (males 1, 3, 7) to either the no-preference class or to a sub-cluster of males with weak preference for WB within the preference for WB cluster.

The stress index of quality of the clustering indicated that the Ward method had the best performance over other clustering algorithms (Tab.1, Appendix B). The Calinski-Harabasz index was maximized for an optimal number of clusters c = 5 with the Ward method (but also using average linkage and centroid method). Its high absolute value (tab. 1 and fig 1, Appendix B) indicates that clusters are well separated and homogeneous. The average method produced the most biologically meaningful clustering in relation to male preferences (Fig. 2, Appendix B). The indices of quality of the *average* clustering and the number of clusters were analyzed via a bootstrap simulation study (Table 2 in Appendix B). Two groups were suggested with low quality (cophenetic: 0.654, stress: 0.461). However, a two-group clustering was rejected in favour of a 4-group cluster structure given the observed means and 97.5% percentiles generated by the bootstrap study. This classification of individual males by mating preference type in four groups is also biologically meaningful and consistent with the distribution of compositional geometric means of each group as shown in the bar plot of Fig. 4, main text.

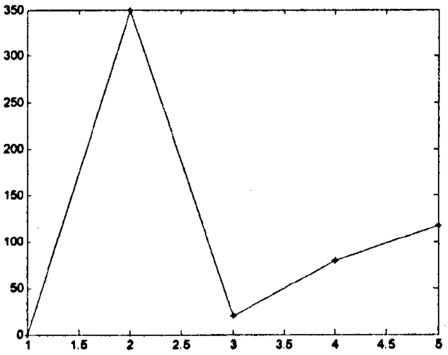
	Clustering algorithm				
	Single	*Complete*	*Average*	*Centroid*	***Ward***
Stress index	1.8255	**0.4595**	0.3498	0.3710	**0.5855**
Number of clusters*	2	**4**	**5**	**5**	**5**

Table 1 Appendix B. Stress index comparing initial distances with cluster-derived distances under different clustering methods (clustering quality highest when stress index = 1). .

* following Calinski-Harabasz method

		Cophenetic correlation	Stress	Calinski & Harabasz	Mojena	Gamma	Point Biserial
	Sample	0.654	0.461	2	2	2	2
Bootstrap	Mean	0.571	0.582	2.834	2.837	6.552	2.790
	Stdev.	0.079	0.062	1.615	0.798	3.346	1.092
	$P_{2.5\%}$–$P_{97.5\%}$	0.379–0.702	0.484–0.733	2–8	2–5	2–10	2–6

Tab. 2 Appendix B. Indices for cluster quality (*cophenetic correlation*, *stress*) and for number of groups (*Calinski & Harabasz's*, *Mojena*, *gamma*, *point biserial*): values across the males and results from the bootstrap analysis (mean, standard deviation, 97.5% and 2.5% percentiles).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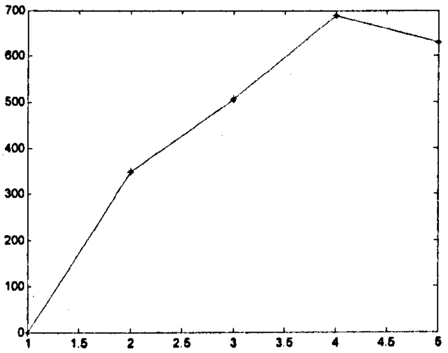
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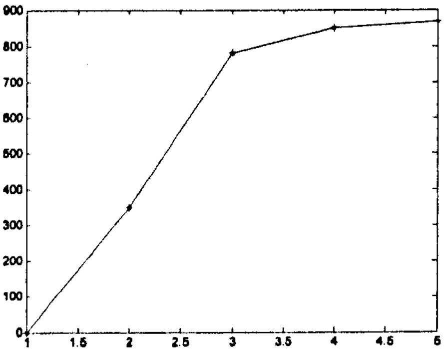
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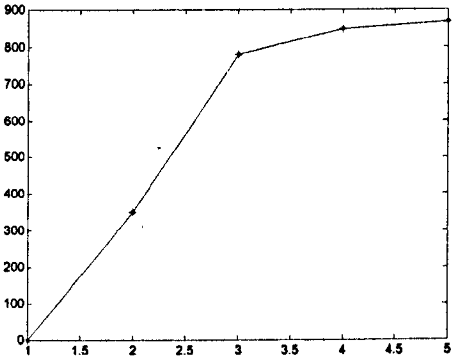
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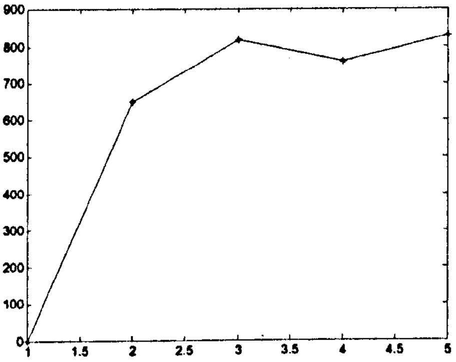
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(E)

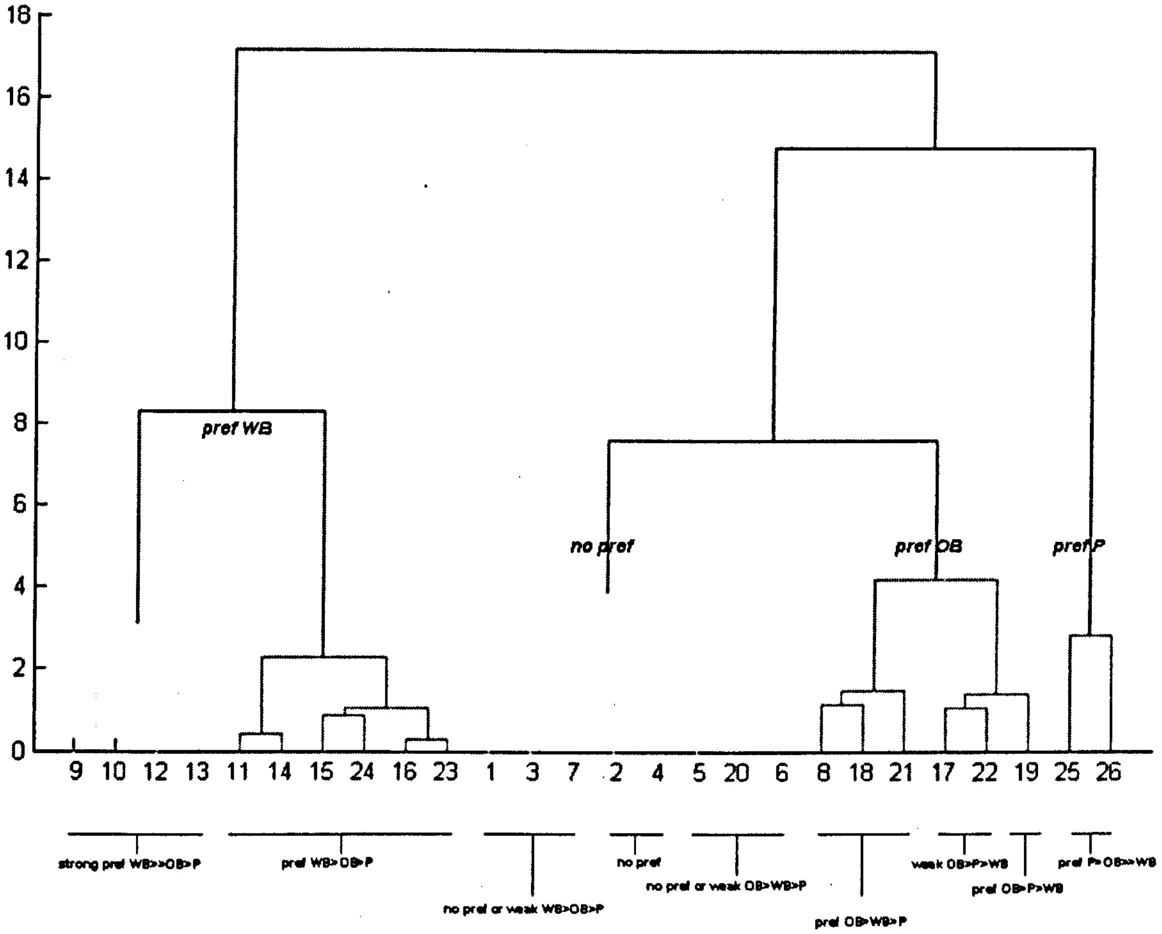

Fig. 2 Appendix B. Dendrogram of individual male mating preferences based on Mahalanobis distance on the compositional dataset and Average linkage method. Clustering identifies four groups of males: two groups of males with strong (*in green*) and weaker (*in violet*) mating preferences for WB females, one group with preferences for OB females (*in red*), for P females (*in blue*) and a group of males with no preference bias to any female morph (*in yellow*).

Appendix C: *Two strategies for multivariate repeatability*

In the univariate case, repeatability can be calculated as

$$R_u = \frac{s_A^2}{s_A^2 + s^2},$$

where s^2_A is the *among-groups* variance component and s^2 is the *within-group* variance component. In the analysis of variance these variance components are calculated from the mean squares between-groups MS_B and within-groups MS_W, as: $s^2_A = (MS_B - MS_W)/n_0$ and $s^2 = MS_W$, where n_0 is related with the sample size per group:

$$n_0 = [1/(G-1)]\left[\sum_1^G n_i - \left(\frac{\sum_1^G n_i^2}{\sum_1^G n_i}\right)\right],$$ n_i = size sample of group i; G = number of groups

Two equivalent expressions of R_u can be deduced:

a) in terms of means squares: $R_u = \dfrac{MS_B - MS_W}{MS_B + (n_0 - 1)MS_W}$,

b) in terms of F = MS_B/MS_W or F ratio: $R_u = \dfrac{F-1}{F-1+n_0}$

For the multivariate case, two generalizations can be considered:

1. From the trace of the variance matrix:

We consider MS_B = Trace(B)/(G-1) and MS_W = Trace(W)/(N-G), where B and W are the matrices of variance 'between' and 'within'; if N the sample size and G the number of groups, we can define the multivariate repeatability as

$$R_m = \frac{\frac{Trace(B)}{G-1} - \frac{Trace(W)}{N-G}}{\frac{Trace(B)}{G-1} + (n_0 - 1)\frac{Trace(W)}{N-G}}$$

as in expression a) for the univariate case. Note that the variability measured by the trace only takes into account the variance of the variables and does not make use of the correlation between them.

2. From the F ratio:

In MANOVA, the Lambda Wilk's statistic $\Lambda = \frac{|W|}{|B+W|}$ is used in the definition of the F statistic $F = \frac{1-\Lambda^{1/s}}{\Lambda^{1/s}}\frac{m_2}{m_1}$, where m_1 and m_2 are the degrees of freedom of a Fisher distribution. This F statistic is known as the Rao's approximation. Here $m_1 = p(G-1)$ and $m_2 = 1+ts-p(G-1)/2$, where

p is the number of variables

G is the number of groups

N is the total sample size

$t = N-1-(p+G)/2$

$$s = \sqrt{\frac{p^2(G-1)^2 - 4}{p^2 + (G-1)^2 - 5}}$$

In the general case, the F statistic has an approximate F distribution. For the particular case when p = 2 (our case in ilr-space), the F statistic has an exact Fisher distribution.

Then, as in expression b) for the univariate case, we define the multivariate repeatability as $R_m = \dfrac{F-1}{F-1+n_0}$.

If one substitutes the value $F = \dfrac{1-\Lambda^{1/s}}{\Lambda^{1/s}}\dfrac{m_2}{m_1}$ in the expression for R_m and applies

$$\Lambda = \frac{|W|}{|B+W|}$$

a new expression in terms of variability is obtained:

$$R_m = \frac{\dfrac{|B+W|^{1/s}-|W|^{1/s}}{m_1} - \dfrac{|W|^{1/s}}{m_2}}{\dfrac{|B+W|^{1/s}-|W|^{1/s}}{m_1} - (n_0-1)\dfrac{|W|^{1/s}}{m_2}}.$$

Here the term $\dfrac{|B+W|^{1/s}-|W|^{1/s}}{m_1}$ has the role of variability *between-groups* and $\dfrac{|W|^{1/s}}{m_2}$ the *within-groups* variability. Note that since the variability is measured with the determinants, both variability and the correlation between variables are reflected in the repeatability.

CHAPTER III

MALE MATING PREFERENCES PRE-DATE THE ORIGIN OF A FEMALE TRAIT IN AN INCIPIENT SPECIES COMPLEX OF LAKE VICTORIA CICHLIDS

Male mating preferences predate the origin of a female trait in an incipient species complex of Lake Victoria cichlids

Michele Pierotti[1] & Ole Seehausen[2]

[1]Department of Biological Sciences, Molecular and Evolutionary Ecology Group, University of Hull, Cottingham Road, HU6 7RX, Hull, UK

[2]Department of Aquatic Ecology and Evolution, Zoological Institute, University of Bern, Baltzerstrasse 6, CH-3012 Bern, and EAWAG Limnological Research Center, Seestrasse 79, CH-6047 Kastanienbaum, Switzerland

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ABSTRACT

Disruptive sexual selection on colour patterns has been suggested as a major cause of diversification in the cichlid species flock of Lake Victoria. In *Neochromis omnicaeruleus*, a colour and sex determination polymorphism is associated with a polymorphism in male and female mating preferences. Theoretical work on this incipient species complex found conditions for rapid sympatric speciation by selection on sex determination and sexual selection on male and female colour patterns, under restrictive assumptions. Here we test the biological plausibility of a key assumption of such models, namely, the existence of a male preference against a novel female colour morph before its appearance in the population. We show that most males in a population that lacks the colour polymorphism exhibit a strong mating preference against the novel female colour morph and that reinforcement is not a likely explanation for the origin of such male preferences. Our results show that a specific condition required for the combined action of selection on sex determination and sexual selection to drive sympatric speciation is biologically justified. Finally, we suggest that Lake Victoria cichlids might share an ancestral female recognition scheme, predisposing colour monomorphic populations/species to similar evolutionary pathways leading to divergence of colour morphs in sympatry.

Keywords

male preference, sexual selection, mate choice, pre-existing preference, *Neochromis*, Lake Victoria, cichlid, sympatric speciation, sex ratio distorter, sex determination

INTRODUCTION

The explosive radiation of cichlid fishes in Lake Victoria provides ideal model systems to test the hypothesis that polymorphisms in mate preferences may cause strong premating isolation and rapid sympatric speciation with very small genome-wide differentiation. More than 500 haplochromine cichlid species have radiated from few ancestral populations within the past 100,000 years (Seehausen *et al.*, 2003; Verheyen *et al.*, 2003), and most of them perhaps within merely 14,600 years (Johnson *et al.*, 1996; Nagl *et al.*, 2002; Seehausen, 2002). Both in Lake Victoria and Lake Malawi cichlids, there is evidence that behavioural mate choice based on colour patterns is often the only isolating mechanism in sympatry (Holzberg, 1978; Marsh *et al.*, 1981; Knight *et al.*, 1998; Seehausen *et al.*, 1997; Seehausen & van Alphen, 1998; Seehausen *et al.*, 1998b; Knight & Turner, 2004).

Several theoretical models have emphasised the potential for sexual selection to drive rapid allopatric (Lande, 1981; Kiester *et al.*, 1984; Lande & Kirkpatrick, 1988; Iwasa & Pomiankowski, 1995; Gavrilets, 2000), parapatric (Lande, 1982; Kirkpatrick & Servedio, 1999; Kirkpatrick, 2000) or sympatric speciation (Wu, 1985; Turner & Burrows, 1995; Payne & Krakauer, 1997; van Doorn *et al.*, 1998; Higashi *et al.*, 1999; Kawata & Yoshimura, 2000; van Doorn & Weissing, 2001; Lande *et al.*, 2001; Gavrilets & Waxman, 2002; Takimoto, 2002; van Doorn *et al.*, 2004). However, empirical evidence is still scarce, and the generality and/or the biological plausibility of conditions under which speciation is observed in such models is much debated (Turelli *et al.*, 2001; Kirkpatrick & Ravignè, 2002; Kirkpatrick & Nuismer, 2004; Arnegard & Kondrashov, 2004).

Lande *et al.* (2001) have proposed that multiple speciation events may occur when sex ratio selection and sexual selection by mutual mate choice interact. The authors model the invasion of a dominant female determiner in a male heterogametic system. This might be a relatively frequent scenario in highly structured populations where mild inbreeding favours female–biased population sex ratios (Bull, 1983; Werren *et al.*, 2002). Lande *et al.*'s models lead to the evolution of a new species carrying the dominant female determiner and an autosomal suppressor that restores the original male heterogamety. There is evidence that master sex determiners are not conserved across even closely related taxa (Saccone *et al.*, 2002; Volff *et al.*, 2003; Peichel *et al.*, 2004) and recent work suggests rapid evolution of new sex determining systems by sequential upstream addition of new master switches to a relatively conserved regulatory pathway (Wilkins, 1995; Zarkower, 2001; Schartl, 2004).

A novel sexually selected trait physically linked to the female determiner could invade since it would be limited to females and therefore protected from sexual selection by female choice. In Lande *et al.* (2001), the novel trait acts as a marker for the dominant female gene and this triggers the evolution of individual male mating preferences for carriers of the dominant female determiner. Another appealing feature of these models of speciation by sexual selection is that genetic variation in mating preferences is not maintained by mutation or by external factors such as sudden shifts in the environment affecting sexual communication, as in most other models of sympatric speciation, but by means of disruptive selection (van Doorn *et al.*, 2004).

These speciation models were inspired by Seehausen *et al.*'s (1999a) study on the ecology and the genetics of colour and sex determination in *Neochromis omnicaeruleus*, a member of the rapidly speciating flock of Lake Victoria cichlids. In this species, some populations are polymorphic for male and female colouration and different colour morphs have identical microdistributions with no morphological or ecological differentiation (Seehausen & Bouton, 1997; Seehausen *et al.*, 1999a). Three colour morphs can occur sympatrically: i. individuals exhibit dark vertical bars on the flanks, on a blueish (males) or a yellow-brown (females) background (P morph); alternatively, ii. vertical bars are disrupted and appear as black blotches on a blueish (males) or white-yellow (females) background (WB morph); iii. males and females exhibit black blotches on a pink to orange background (OB morph). Intermediate phenotypes exist, but are rare. Morph frequencies vary between populations and most populations lack the WB morph altogether. Similar male and female colour polymorphisms have been observed among other species of Lakes Victoria, Kivu and Malawi (Snoeks, 1994; Konings, 1995; Seehausen, 1996; Seehausen *et al.*, 1999b; Lande *et al.*, 2001).

In *N. omnicaeruleus*, the genes associated with both blotched phenotypes (WB, OB) are linked to dominant female determiners (W) on the X chromosome. Their dominance over Y can be counteracted by autosomal suppressors (male rescue genes) in the absence of which female sex-biased clutches are produced (Seehausen *et al.* 1999a). Blotched males are therefore carriers of both the blotch-linked sex ratio distorter and its autosomal suppressor. In colour polymorphic populations (e.g. Makobe Island population), males with the rescue gene were found to exhibit mate preferences for blotched females (WB/OB), whereas males without suppressor preferred females (P) lacking the dominant female determiner. The resulting selective mating among colour morphs led Seehausen *et* al. (1999a) to suggest that the *N. omnicaeruleus* polymorphism has properties of an incipient stage in sympatric speciation by sex ratio selection and disruptive sexual selection on male and female colouration.

Despite their potential to provide new insight in the role sex-linked genes and sex determination might play in the generation of new species, Lande *et al.*'s (2001) models rely on a restrictive assumption regarding the mate preference state of the ancestral population invaded by the novel sex-linked female colour. Counter intuitively, a male mating preference against the novel female colour is required to be present before the appearance of the female colour in the population. Such pre-existing preference allows the creation of a pool of females of the novel colour avoided by the majority of males but available to rare mutant males that mate with such females sustaining less intense competition for mating opportunities. The resulting sexual selection on male mating preferences is necessary to obtain partial association between novel W-linked colour allele and novel male preference. However, this assumption may be problematic since there is no *a priori* reason to expect the presence of male mate choice in lekking species with highly skewed parental investment (Trivers, 1972; Parker, 1983; Kokko & Johnstone, 2002) such as the maternal mouthbrooding cichlids of Lakes Victoria and Malawi.

Male mating preferences against novel phenotypes in monomorphic populations may evolve to prevent hybridization with sympatric species (reinforcement; Dobzhansky, 1940; Noor, 1999). Alternatively, such male mating preferences could either represent an ancestral trait retained in a species that is a member of a recently radiated clade, or could have evolved by direct natural selection on mating preferences (Servedio, 2001; Albert & Schluter, 2004) or as a by-product of competition and ecological character displacement (Rundle & Schluter, 1998; Schluter, 2000, 2001).

Seehausen *et al.* (1999a) did find male preferences for and against the blotched female types in a colour polymorphic population of the Lake Victoria haplochromine *Neochromis omnicaeruleus*, however these might have evolved after the polymorphism appeared, e.g. under direct selection for genetic compatibility. Males susceptible to the sex ratio distorter would be selected to avoid mating with female carriers (blotched morphs WB and OB) and instead to prefer mating with the yellow-brown female morph (P); resistant males would preferably mate with female carriers (WB/OB). If this scenario is correct, we do not expect P monomorphic populations to harbour male mating preferences for or against P /WB female colour morphs.

Here we consider a colour monomorphic population (ancestral population in Lande *et al.*'s model) of the cichlid *Neochromis omnicaeruleus* and test whether there is any evidence of individual male mating preferences against (or for) a novel female colour morph prior to its occurrence in the population and whether any such preference could be the result of reinforcement of pre-zygotic reproductive isolation against sympatric closely related species. We find that most males show a strong mating preference against the novel female morph (WB), and that males that prefer the ancestral P over the novel WB females also prefer heterospecific P females over conspecific WB females. We conclude that a key assumption of Lande *et al.*'s (2001) models of sympatric speciation by selection on sex determination and mating preferences reflects real states of natural populations. We suggest that male Lake Victoria cichlids share a non-specific ancestral female recognition scheme that might allow repeated generation of male mating preference polymorphisms in sympatry, in different populations and in different cichlid species, potentially leading to sympatric speciation, as modelled by Lande *et al.* (2001).

MATERIALS AND METHODS

Study species

We conducted our experiments on wild caught *N. omnicaeruleus* males from the P monomorphic population of Ruti Island, Western Speke Gulf, Lake Victoria. Female pairs in conspecific trials and control males were wild caught individuals from the colour polymorphic population of Makobe Island, which has been studied in the past for the presence of polymorphisms in male and female colouration, sex determination system and mate preferences (Seehausen *et al.*, 1999a,b). A WB morph has never been observed at Ruti Island among more than 400 fishes examined over more than a decade of sampling (Lande *et al.*, 2001, Seehausen, additional unpubl. data collected between 2000 and 2004). In the Makobe Island population, more than 16% of the individuals belong to the WB morph and Seehausen *et al.* (1999a) showed that males from that population are polymorphic for individual male preferences for female colour morphs. As a control group for the Ruti Island sample (colour monomorphic population), males from the colour and male preference polymorphic population of Makobe Island, were tested with the same design.

For the conspecific/heterospecific trials, we used WB females from the Makobe Island colour polymorphic population of *Neochromis omnicaeruleus* and P females from the Ruti Island colour monomorphic population of *Neochromis* 'yellow anal scraper' (Seehausen, 1996). *N.* 'yellow anal scraper' differs very little in head morphology and body shape from *N. omnicaeruleus* but the two species have very distinct male nuptial colouration and coexist at several sites, including Ruti Island

(Seehausen, 1996). Female *N.* 'yellow anal scraper' are yellow-brown with dark vertical bars and very similar in colour pattern to *N. omnicaeruleus* females. If male mating preferences in *N. omnicaeruleus* for P females of their own species have evolved by reinforcement of reproductive isolation, males are expected to avoid mating with P females of the sympatric closely related species, *N.* 'yellow anal scraper'.

Housing conditions

Males were collected at Ruti Island and Makobe Island, Southern Lake Victoria, and shipped to the laboratory in Hull where they were kept in large population tanks. A few months prior to the start of the experiment and for the entire duration of the experiment, males were kept into individual 19x19x18cm aquaria allowing no visual contact with either females, or with heterospecifics with female-like colour patterns. Individual females were kept in transparent plastic enclosures inside larger tanks. The enclosures were identical to the ones used in the trials. All aquaria were maintained at 26 ±2°C and illuminated with daylight fluorescent bulbs on a 12:12h light:dark cycle. Fish were fed twice a day with flake food and a vitamin-enriched mix of mashed prawns and peas.

Experimental design

We simultaneously presented *N. omnicaeruleus* males from the colour monomorphic population of Ruti Island with a yellow-brown (P) female and a black and white blotched (WB) female, both from the colour polymorphic population of Makobe Island. By using both female morphs from the same island, which is not the island the males came from, we control for possible 'own population' effects on male mating preferences. The number of lateral displays towards females was used as a measure of male courtship intensity. Lateral displays are widely used as the standard measure of cichlid courtship behaviour (e.g. Baerends & Baerends, 1950; Carlstead, 1983; McElroy & Kornfield, 1990; Seehausen & van Alphen, 1998; Maan *et al.*, 2004). A standardized mean preference score was calculated as the ratio (lateral displays to P female – lateral displays to WB female)/total number of lateral displays.

Lande *et al.*'s (2001) model requires the presence of individual males with consistent male preferences for female colouration. Each experimental male was tested with 6-8 different female pairs, and Wilcoxon signed rank tests were performed on the number of lateral displays to determine whether there were consistent individual preferences for either female colour morph. Wilcoxon signed rank tests on mean standardized lateral display scores of each male were used to compare direction and strength of male preferences between conspecific and heterospecific choice trials. No difference between female colour morphs was identified with respect to standard length, weight or gravidity stage in female pairs (Wilcoxon signed rank tests: all n.s.). We performed one-sample *t* tests on the arcsin-transformed mean preference scores for the two separate male populations to test for deviation from the null expectation of no preference.

Mate choice trials

Individual males were given the choice between a P morph female and a WB morph female each placed in a transparent plastic enclosure at either end of a 100x40x40cm experimental aquarium. Males were introduced into the experimental aquarium and left to acclimatize overnight. The following day, a yellow-brown barred female (morph P) and a blotched female (WB) were placed each in a plastic enclosure. For each trial a different set of females was used and every male was only tested once with each female. Males were left in the experimental tank between trials, and were tested with no more than 3 female pairs a day. The left and right positions of P and WB females were rotated randomly between trials. Male behaviour was video recorded and scored for 20 minutes from its first interaction with a female, defined as approaching the female to a distance of less than 20cm. Trials in which the male did not interact with either female, were discarded. After a trial, standard length (to the nearest 0.5mm) and weight (to the nearest 0.1g) were measured for each female. Gravidity stage was also assessed using a 1-5 score as follows: 1. immature/recently spent, 2. early ripening, 3. ripening, 4. fully ripe, 5. spent.

RESULTS

We conducted 45 conspecific morph choice trials using seven wild caught males from the colour monomorphic Ruti Is. population (R1-R7) and 52 conspecific morph choice trials on seven wild caught males of the colour polymorphic Makobe Is. population (M1-M7).

When tested with a conspecific female colour morph pair (P vs WB) from a polymorphic population (Makobe Island; open bars in fig.1), six Ruti Island males had consistent mate preferences for the P over the WB female colour morph, one male (R1) did not show significant preferences, whereas none showed preference for females of the WB morph (Ruti Island males R1-R7: Wilcoxon signed rank tests: table 1 and figure1, shaded bars). An overall *P*-value, obtained from the individual Wilcoxon signed rank tests, using Fisher's combined probability test, was highly significant ($\chi^2 = 43.110$; df = 14; $P < 0.0005$).

In contrast with the results for Ruti Island males and in line with a previous study (Seehausen *et al.* 1999a), males from the colour polymorphic population (Makobe Is.) exhibited individual variation in the direction of male mating preferences for female colour morph, with all three preference conditions, i.e. preference for P (male M1), preference for WB (males M3, M5-M7) and no preference (males M2, M4), represented (Wilcoxon signed rank tests: table 1 and fig. 1, open bars). Male preferences were significantly different from zero in the colour monomorphic Ruti population ($t = 6.054$, $P = 0.001$) but not significantly different from zero (i.e. no preference) in the colour polymorphic Makobe population ($t = -1.508$, $P = 0.182$).

To explore the possibility that reinforcement or reproductive character displacement is responsible for Ruti Is. male preferences for the P female morph, we ran 12 conspecific/heterospecific choice trials on six Ruti Is. males (R1-R5, R7), testing them with P females of the sympatric *Neochromis* 'yellow anal scraper' versus conspecific *N. omnicaeruleus* WB females from the polymorphic population (Makobe Is.). Ruti Is. males preferred the heterospecific P female over the conspecific WB female. Male preferences in conspecific/heterospecific choice trials did not differ from those in conspecific choice trials (Wilcoxon signed rank test on mean preference scores: n = 6, z = - 0.105, *P* = 0.917; fig. 2).

No significant differences were found in male courtship with regard to female size (standard length), weight or gravidity stage (Wilcoxon signed rank tests, all n.s.).

Ruti Island (monomorphic) population					**Makobe Island (polymorphic) population**				
Male	**preference**	**Z**	**P**	**N**	**Male**	**preference**	**Z**	**P**	**N**
R1	no pref	-1.05	0.29	6	M1	P	-2.201	0.028	6
R2	P	-1.99	0.046 *	6	M2	no pref	-0.070	0.944	8
R3	P	-2.03	0.042 *	8	M3	WB	-2.197	0.028	7
R4	P	-2.21	0.027 *	7	M4	no pref	-1.352	0.176	8
R5	P	-2.04	0.041 *	6	M5	WB	-2.371	0.018	7
R6	P	-2.21	0.027 *	6	M6	WB	-2.033	0.042	8
R7	P	-2.23	0.026 *	6	M7	WB	-2.524	0.012	8

Table 1 Z and *P* values of Wilcoxon signed-ranks tests of male mating preference (number of courtship displays). Males R1-R7 are wild caught individuals from the Ruti I. population; males M1-M7 are wild caught individuals from the Makobe I. population.

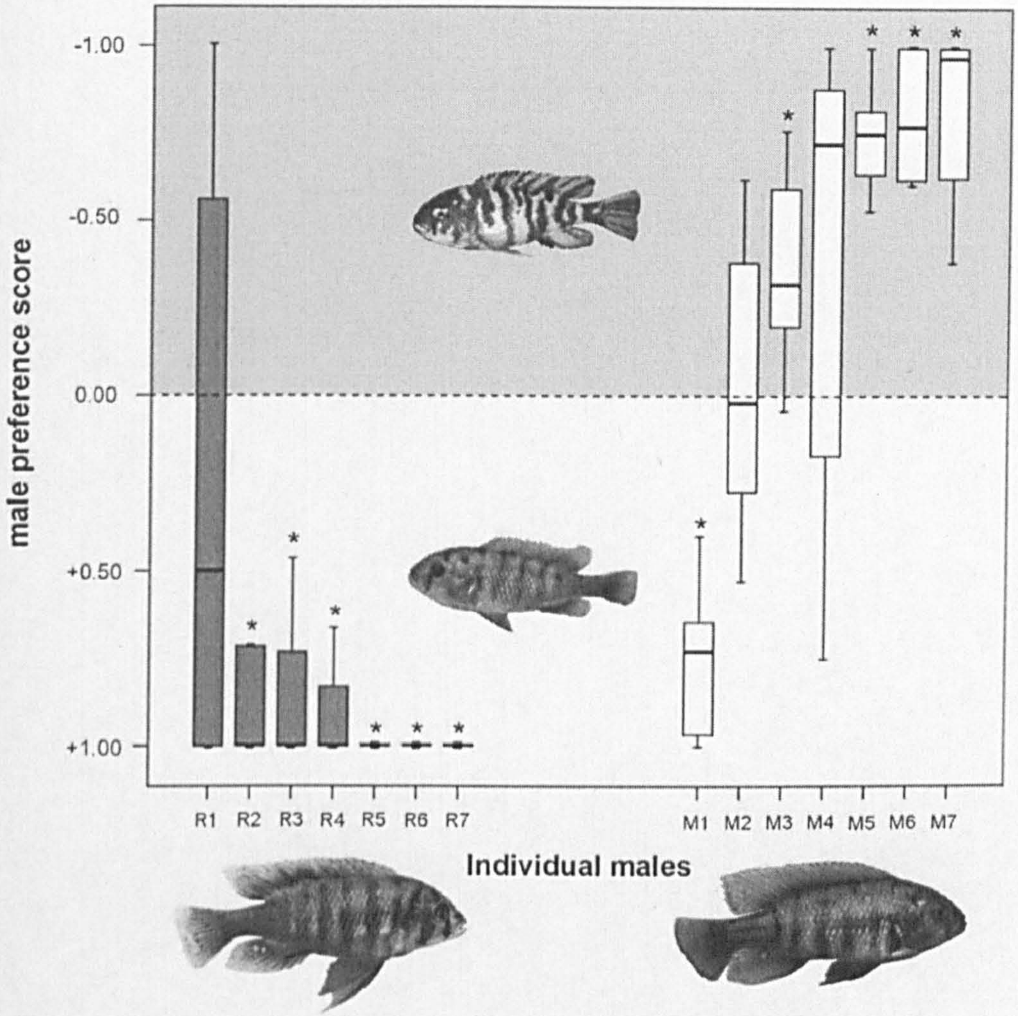

Figure 1 Individual male preference scores (lateral displays to P female – lateral displays to WB female/total number of lateral displays). Positive preference score values indicate male preference for P females, negative values indicate male preference for WB females.

Shaded bars, wild caught Ruti Island males (R1-R7); *Open bars*, wild caught Makobe Island males (M1-M7), both groups tested with P/WB Makobe Island females .

Data are given as boxplot diagrams showing medians (middle line in the boxes), first and third quartiles (boxes) and range (whiskers). Outliers (◦) are also shown. Significance levels are derived from *p*-values based on Wilcoxon signed-ranks tests on male choice trials: * $P<0.05$; (see also table 1).

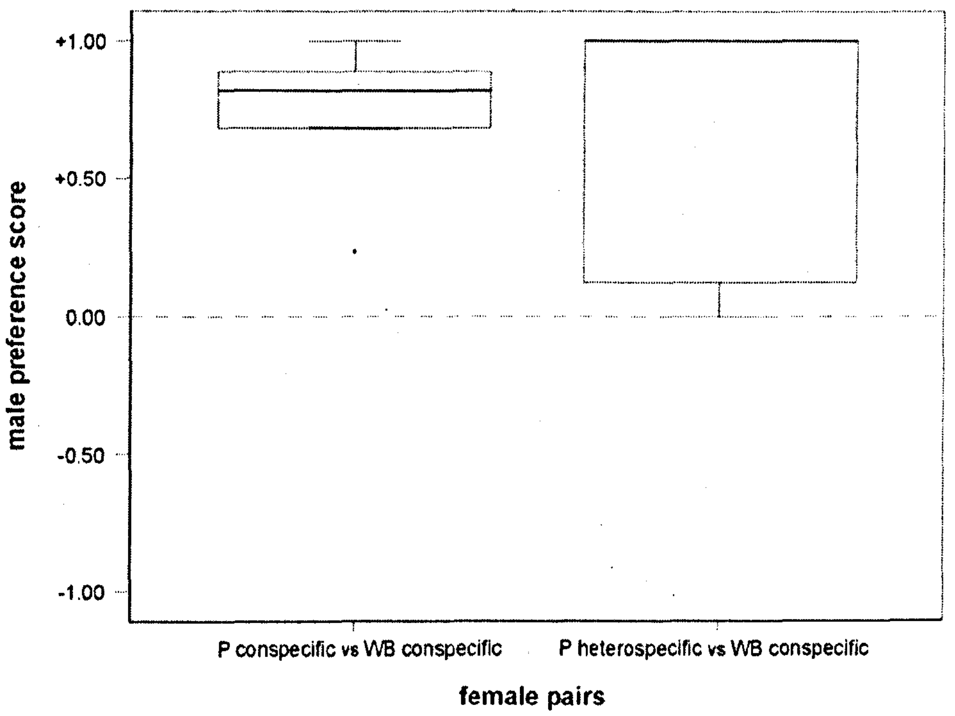

Figure 2 Male preference scores in conspecific choice trials (*N. omnicaeruleus* Makobe Is. females of P and WB colour morph) and in conspecific/heterospecific choice trials (*N.* 'yellow anal scraper' Ruti Island females of P morph and *N. omnicaeruleus* Makobe Island females of WB morph). Data given as in fig.1.

DISCUSSION

Our results are consistent with the hypothesis that a male mating preference for P females in *Neochromis omnicaeruleus* predates the origin of female colour polymorphism. We have shown that individual males in a colour monomorphic population (Ruti Island) have significant preferences against females of a novel colour. In contrast, males of a colour polymorphic population (Makobe Island) showed variation in male preferences for or against the alternative female colour morphs, confirming results of an earlier study (Seehausen *et al.*, 1999a). The novel male mating preference for females of the WB morph, observed in some males of the polymorphic population of Makobe Island, is therefore likely to have evolved by selection on male mating preferences. There is evidence that male mating preferences for female colouration have a genetic basis in the Makobe Island population of *N. omnicaeruleus* (Seehausen *et al.*, 1999a; Seehausen & Brandsen, unpubl. data) and in the Malawi cichlid *Pseudotropheus zebra* 'gold' (Pierotti *et al.*, unpubl. data). Consistent with the potential for male preferences to evolve, we observed variation in the strength of mating preference among the Ruti Island males. These results lend support to the speciation pathways envisaged by Lande *et al.* (2001), showing the biological plausibility of a critical model assumption, and suggesting that a condition of ancestral pre-existing male preferences might be a shared state among the rockdwelling cichlid species of Lake Victoria.

Males from Ruti Island use female colouration to identify potential partners: when given the choice between a conspecific blotched female (WB morph) from an allopatric population and a heterospecific yellow-brown female (P morph) from a sympatric species, males expressed a preference for the sympatric heterospecifics. Together with the fact that yellow-brown colouration is widespread in females of the haplochromine cichlid species flock of Lake Victoria (Greenwood, 1974, 1981; Seehausen, 1996) and represents most of the female fish community at Ruti Island, this suggests that male preferences are unlikely to be the product of reinforcement of reproductive isolation against closely related species. Instead it seems likely that a mating preference for a female colour that is shared among most species in Lake Victoria represents an element of an ancestral mate recognition system. This may have evolved by direct selection or by reproductive character displacement against distantly related species prior to the adaptive radiation in Lake Victoria, or at least prior to the radiation of *Neochromis*. The riverine ancestors of the Lake Victoria cichlids lived in sympatry with other distantly related cichlids of the genera *Astatoreochromis, Pseudocrenilabrus* and *Oreochromis*. The females of these species differ in colouration, and hybrids between Lake Victoria cichlids (and most likely their common ancestor) and these cichlids are probably inviable (F. Witte, pers. comm. for *Astatoreochromis*; A. Smith, pers. comm. for *Oreochromis*) or at least infertile. Therefore, a preference against blotch may be the by-product of selection on mate recognition in the ancestral population that gave rise to the Lake Victoria radiation. By contrast, hybrids between species that belong to the radiation are fully fertile (Crapon de Caprona, 1986; Crapon de Caprona & Fritzsch, 1984; Seehausen *et al.*, 1997), as is not surprising given the extreme young age of the radiation. This makes a recent origin by reinforcement of male mating preferences for yellow-brown females in *Neochromis* an implausible scenario. There is growing evidence for the effects of historical processes on mating preferences across speciation events (Ryan *et al.*, 2001). For example, in some *Drosophila* species female preferences for ancestral traits can persist in descendant populations where such preferences apparently lack any function (Kaneshiro, 1983). The gynogenetic fish *Poecilia formosa*, originated by hybridization about 10,000 to 100,000 years ago (Avise *et al.*, 1991), shows female preferences, in the absence of any male genetic contribution to the offspring, that derive from its sexual parental species, *P. latipinna* and *P. mexicana* (Marler & Ryan, 1997).

An alternative explanation for the presence of male mating preferences in the *N. omnicaeruleus* monomorphic population of Ruti Island, involves effects of blotched colouration. A black and white blotched (WB) colour morph is not known for the Ruti Island population but an orange mottled morph, the heterozygous form of orange blotch (OB), was present in catches of the years 1991-1992

(0.6% frequency), but was never again seen in samples of several hundred females taken in the years 1993, 1995, 1996, 2000, 2002 and 2003 (Seehausen, unpubl. data). We do not know whether *N. omnicaeruleus* males distinguish between the different female base colours, i.e. yellow-brown (P morph), orange (OB morph), or yellow-whitish (WB morph), or whether they rely on the regular black bar pattern in P females as opposed to the disrupted pattern (blotched or mottled) in WB and OB females, or a combination of the two criteria. If orange-blotched and white-blotched colourations are equivalent for males, then the evolution of male preferences against WB females (i.e. for P females) could have been driven by the presence of an orange-blotched morph in this population in the past. In this scenario, a male preference against blotch could have evolved to avoid female biased broods and perhaps, reduced clutch size due to the production of inviable YY individuals. Following the extinction of blotched morphs at Ruti Island, a preference for blotch would possibly be selected against. A male preference for plain females, on the other hand, would probably not be costly, and would more likely have been retained. Therefore, a past invasion/extinction of blotch would prepare the conditions for a second invasion, this time triggering Lande *et al.*'s evolutionary dynamics, leading to rapid sympatric speciation.

Regardless of the historical path that led to the origin of male mating preferences in the Ruti Island population, our results indicate that the assumption of a pre-existing male mating preference, which is required for Lande *et al.*'s model of sympatric speciation by selection on sex reversal and sexual selection, is biologically realistic. If a blotch-linked dominant female determiner now invaded a population monomorphic for female colouration and male mating preferences such as the Ruti island population we studied (e.g. by dispersal of blotched individuals from Makobe Island, 10 km away), we might witness the development of similar polymorphisms in sex determination, mate preferences and both male and female colour patterns, leading to assortative mating between colour morphs and potentially divergence in sympatry.

The brown-barred/blotch colour polymorphism is well represented in both Lake Victoria and Lake Malawi rockdwelling cichlid radiations (Greenwood, 1981; Konings, 1995; Seehausen, 1996) and a polymorphism in male mating preferences for either brown barred (P) or blotched (OB) female colouration has been found in the colour polymorphic cichlid *Pseudotropheus zebra* 'gold', a member of the Lake Malawi cichlid species flock (Pierotti *et al.*, unpublished data). Knight &Turner (1999) showed that males of *P. zebra* 'gold' and of a sympatric closely related species were unable to distinguish conspecific from heterospecific females of the same colour morph, suggesting that reinforcement is unlikely to be responsible for male preference evolution in the *P. zebra* species complex. Although our experiment does not specifically address this issue, we suggest that male Lake Victoria (and possibly Lake Malawi) haplochromine cichlids might share a non-specific ancestral female recognition scheme (brown with dark vertical bars). Future studies should specifically test this possibility: if confirmed, this would suggest that any population/species monomorphic for female colouration (and male mating preferences) might be prone to the evolution of male mating preference polymorphisms in sympatry, following invasion by blotch-linked female determiners, and might diverge in sympatry, as modelled by Lande *et al.* (2001). Moreover, although these models were inspired by a particular colour polymorphism, there is no reason why any other novel female trait, acting as a marker for a new sex determiner, could not trigger similar speciation dynamics.

There is growing evidence that sex ratio distorters may be more widespread than previously assumed (Hurst & Pomiankowski, 1998) and that the nature of the selfish elements involved is likely to determine whether male, female or mutual mate preferences evolve. Cytoplasmatic factors are thought to be responsible for male mate choice in the butterfly *Acraea encedon* (Randerson *et al.*, 2000); sex ratio meiotic drive genes have shaped female preferences in stalk-eyed flies (Wilkinson *et al.*, 1998a,b; Lande & Wilkinson, 1999); dominant X-linked female determiners have triggered the evolution of male and female mating preferences in the Lake Victoria cichlid fish *N.*

omnicaeruleus. The blotch polymorphism, widespread in the haplochromine cichlids of Lakes Victoria and Malawi (Kocher, 2004), can provide new insights in the interplay between sex ratio distorters, sexual selection, and the evolution of reproductive isolation. Perhaps this interplay is at the heart of rapid speciation events in the cichlid flocks of the East African Lakes.

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CHAPTER IV

VARIATION AND MODE OF INHERITANCE OF MALE MATING PREFERENCES FOR FEMALE COLORATION IN A POLYMORPHIC LAKE MALAWI CICHLID SPECIES

Variation and mode of inheritance of male mating preferences for female coloration in a polymorphic Lake Malawi cichlid species

Michele E.R. Pierotti[1]*, Mairi E. Knight[2], Simone Immler[3], Nicola Barson[4], George F. Turner[1], Ole Seehausen[5,6]

[1] Molecular Ecology & Evolution Group, Department of Biological Sciences, University of Hull, Cottingham Road, Hull, HU6 7RX, UK

[2] Terrestrial Ecology Research Group, School of Biological Sciences, University of Plymouth, UK

[3] Evolution & Behaviour Research Group, Department of Animal and Plant Sciences, University of Sheffield, UK

[4] Biodiversity & Ecological Processes Research Group, School of Biosciences, University of Cardiff, UK

[5] Aquatic Ecology and Macroevolution, Institute of Zoology, University of Berne, Switzerland

[6] EAWAG Center of Ecology, Evolution and Biogeochemistry, Kastanienbaum, Switzerland

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ABSTRACT

Female color polymorphisms are common in the cichlid species radiations of Lake Victoria and Lake Malawi. According to theory, when a population harbors variation in sex determining factors, polymorphism in female-linked coloration might generate individual variation in male mating preferences for female color morphs. We tested whether individual males exhibit consistent mating preferences for female color morphs in the Lake Malawi cichlid *Pseudotropheus* (*Maylandia*) 'zebra gold', a species polymorphic for female coloration and sex determination. We also explored whether male mating preferences could be predicted by maternal coloration or were acquired by imprinting on siblings' coloration. We found large individual variation in the strength and direction of male preferences for sex-linked female color patterns. Male mating preferences could be predicted by the mother's color morph and were not affected by visual imprinting. Our findings represent the first evidence of male choice on sex-linked female coloration in a Lake Malawi cichlid. We discuss the observed large individual variation in the direction and strength of male mating preferences in the context of recent models of speciation by selection on sex determining genes and sexual selection.

keywords

male preferences, speciation, cichlid, mate choice, sex ratio, sex determination

INTRODUCTION

The study of within-population variation in sexually selected traits has a long tradition dating back to Darwin (Andersson 1994; Darwin 1871). It is then somewhat surprising that within-population variation in mate preferences has not followed a similar fate. Individual variation is a prerequisite for selection to act on preferences and can have profound consequences on the intensity and direction of sexual selection (Jennions and Petrie 1997; Wagner 1998). Moreover, within-population variation in mate preferences has important bearing on speciation studies. Many models of sympatric speciation by sexual selection assume pre-existing mate preference variation in the population. In some cases (e.g. Higashi et al. 1999; Takimoto et al. 2000; Turner and Burrows 1995), open-ended female preferences of opposite direction and similar strength are required for speciation to occur. However, as pointed out by Arnegard and Kondrashov (2004), there is little empirical evidence in support of these models' assumptions, given that female mating preferences mediating disruptive selection have been rarely demonstrated in natural populations (Greene et al. 2000; Morris et al. 2003; Sappington and Taylor 1990).

In a recent speciation model (Lande et al. 2001; Kocher 2004), not female but male mating preferences play a pivotal role. The authors showed that male-male competition in female color polymorphic species can trigger the evolution of male mating preference variation for female coloration. When female color morphs are associated with different sex determining factors, as in the XYW system of the platyfish (Orzack et al. 1980) and in various haplochromine cichlids (Seehausen et al. 1999; table in Lande et al. 2001), the interaction between sex factors of different strength and individual variation in male mating preferences for female 'color and sex factor' morphs can lead to linkage disequilibrium between color, sex factor and male preferences, and eventually drive sympatric speciation.

The model was inspired by Seehausen et al.'s (1999) study on the Lake Victoria cichlid *Neochromis omnicaeruleus*. In this species three color morphs can co-occur at various frequencies in different populations. One color morph (plain morph, P) shows the typical pattern of dark vertical bars common in many other rock-dwelling cichlids of Lakes Victoria and Malawi. In the other two morphs, vertical bars are substituted by black blotches on an orange background (orange-blotched morph, OB) or on a yellow-white background (piebald morph, WB). The authors showed that both blotched morphs are carriers of different dominant female determiners (sex reversal factors) linked to the blotch genes. As a consequence, matings between P and blotched (WB/OB) individuals result in heavily female biased broods. Seehausen et al. (1999) found male and female mating preferences for the different color morphs and concluded that *N. omnicaeruleus* might represent a case of arrested incipient speciation by selection on sex reversal and sexual selection.

Male mating preferences, as found in *N. omnicaeruleus*, are not expected to be common among the haplochromine cichlid fishes of Lakes Victoria and Malawi. The haplochromine cichlids of the East African lakes are polygynandrous female mouthbrooders: while male investment is limited to courtship and gamete production, females incur high costs of parental care during the mouthbrooding period, when they refrain from feeding and are possibly more exposed to predators. Given such highly asymmetric investment, strong female choice is expected but the reverse seems unlikely. Male choice for female ornamentation in non sex-role reversed fish species is rarely described (Amundsen and Forsgren 2001, 2003; Basolo and Delaney 2001; Craig and Foote 2001) but this might also be partly due to a research bias (Houde 2001).

To date, there is no evidence that female sex-linked color polymorphisms are the target of mate choice in Lake Malawi cichlids or that individual variation exists in male mating preferences for alternative female color morphs, as invoked by Lande et al.'s (2001) model of sympatric speciation.

The mechanism of mate preference inheritance can influence the evolutionary trajectory of a sexually selected trait and possibly the likelihood and speed of species divergence by sexual selection (Albert 2005; Grant and Grant 1997; Laland 1994; Lachlan and Servedio 2004; Price

1998; Verzijden et al. 2005). Cichlid fishes have been the object of intense research in sexual selection and while evidence for genetic inheritance of mate preferences has only recently started to emerge (Haesler and Seehausen 2005), the existence of sexual imprinting in cichlids has long been invoked. Earlier studies reported an influence of parental color morph on female mate choice in the neotropical cichlid *Archocentrus nigrofasciatus* (Siepen and Crapon de Caprona 1986; Weber and Weber 1976). Siblings' coloration seems also to be implicated in the development of female mating preferences in *Hemichromis bimaculatus* (Kop and Heuts 1973), in *Astatotilapia burtoni* (Sjölander and Fernö 1973) and in *Archocentrus nigrofasciatus* (Weber and Weber 1976). In the Lake Malawi *mbuna* cichlids, there is no parent-offspring interaction after the offspring leave the mother's mouth. Once released, fry rush to the nearest natural shelter to avoid predation (Genner and Turner 2005).

Therefore there is very little opportunity for fry to imprint on mother's color patterns. However, imprinting on sibs' coloration might take place in this early stage of free swimming.

We studied male mating preferences in the blotch polymorphic *Pseudotropheus (Maylandia)* 'zebra gold', a species belonging to the Lake Malawi cichlid flock. Previous work has established within-population variation in sex determining factors and the association between dominant female sex determiners with dominant blotch coloration in this species (Holzberg et al. 1978; Knight 1999). Here we tested whether male mating preferences for female color morphs have evolved in this species and whether there is individual variation in strength and direction of mating preferences, as required in models of sympatric speciation by sexual selection (Higashi et al. 1999; Lande et al. 2001). We also examined the mechanisms of preference acquisition by testing whether male mating preferences have a genetic basis or are acquired by imprinting on sibs' coloration.

MATERIALS AND METHODS

Study species

Pseudotropheus (Maylandia) 'zebra gold' (also known by the junior synonym *Metriaclima;* e.g. Stauffer et al. 1997*)* is an undescribed species of rockdwelling, ephilitic algae scrapers. Two color morphs occur in the population (Nkhata Bay) considered in this study: a 'gold' morph (P), with males gold-yellow with 6-7 dark vertical bars and females brown with dark vertical bars, and an 'orange blotched' morph (OB) with females orange with irregular black blotches; OB males are extremely rare (Konings 2001). Female OB blotched coloration is dominant in *P.* 'zebra gold' and in other haplochromine species (Holzberg et al. 1978; Seehausen et al. 1999; Knight 1999). In *Pseudotropheus* (*Maylandia) zebra*, Streelman et al. (2003) recently identified a single genomic region responsible for OB coloration, in physical linkage with the cichlid proto-oncogene *c-skil* (Huang et al. 1999).

Housing conditions

Experimental males were kept in individual 19x19x18 cm aquaria allowing no visual contact of the test males with either female color morph. Females were kept in a female population tank. All aquaria were maintained at 26 ±2°C and illuminated with daylight fluorescent tubes on a 12:12h light:dark cycle. Fish were fed twice a day with flake food and a vitamin-enriched mix of mashed prawns and peas. Males and females were PIT (Passive Identification Transponder) tagged for individual identification.

A pilot study was conducted in 2001 on 14 individual males to assess whether males sired by P or OB mothers differ in their mating preferences for either female color morph. Seven males sired by wild caught P parents (from a large lab maintained P stock) and seven males sired by wild caught P father and OB mother (from a large lab maintained OB stock) were tested 1-3 times each with a set of 4 P and 4 OB females. Male courtship behavior (see below for details) towards females of each morph was recorded for 15 minutes. During these trials, males and females could freely interact. For this reason, male mating preferences by visual cues could not be disentangled from effects of chemical and possibly acoustic communication.

A restricted maximum likelihood (REML) linear mixed model, with female morph and male pedigree as fixed factors and female group and male identity as random factors, was performed on the arcsine transformed proportions of darts. This suggested a significant interaction between male pedigree and female morph (REML: Wald F = 31.67, $P < 0.001$). A standardized mean preference score was calculated as the ratio (dart displays to P female – dart displays to OB female)/total number of dart displays. A Mann-Whitney test on individual mean display scores showed that males sired by a P mother courted more P females than males sired by a OB mother. ($U = 8.5$, $P = 0.038$).

This preliminary test could not control for the influence of non-visual communication on male choice for females of different color morph. The results might have been due to non-visual male preferences (chemical, acoustic, etc.) or by the interaction of visual and non-visual cues. Moreover, the design did not allow the assessment of individual male mate preferences. Therefore, the preliminary observations prompted us to design an experiment aiming at isolating visual male preferences as well as identifying variation in individual male mating preferences for female coloration. We controlled for potential imprinting on mother's coloration by retrieving fry from their mother's mouth before natural release (see below for details) and imprinting on mother's non visual cues by testing male offspring mating preferences in visual-only designs. Finally, we tested the effects of imprinting on sibs' coloration with a split-brood cross-sib fostering design.

Mode of inheritance of male preference

Experimental males were the sons of P morph fathers and either P morph mothers or OB morph mothers. P morph parents derived from an experimental P line, OB morph mothers from an experimental OB line and were unrelated to individuals used in the pilot study.

To prevent any visual contact between mother and offspring, fry were gently removed from their mother's mouth after a week of incubation, i.e. well before they would be naturally released by the mother (18-24 days). A split-brood cross-sib fostering design was used. Both PxP and OBxP clutches were split in two rearing treatments, resulting in four experimental groups: individuals were exposed to either P sibs only, or to sibs of both morphs. Families from P parents (PxP) contain only individuals with P color patterns, families from heteromorphic pairs (OBxP) are comprised of both "P" and "OB" phenotypes. Fry of P and OB morphs are initially non-blotched and indistinguishable. OB morphs begin developing the first melanin spots only after about 11 weeks post fertilization. For this reason, OB only rearing treatments cannot be made. Offspring were removed from 'P sibs only' treatments, once they showed the first signs of melanin spot development. Blotched fry at the same early stage of color pattern development were added to experimental clutches from PxP parents and assigned to OB+P rearing treatment.

Male choice trials

Two 14-litre aquaria were placed at the short ends of the 2720-litre experimental tank. This was fitted with a heater, kept at 26° C, an external filter and a halved terracotta flowerpot placed in the centre as shelter and spawning site.

Males were introduced into the experimental aquarium and left to acclimatize overnight. The following day, a brown barred female (morph P) and an orange blotched female (OB) were introduced each in one of the two small aquaria. These were then covered with a lid to exclude water or spray being exchanged between compartments. Therefore, no chemical communication was allowed between experimental male and females during the trials.

For each trial a different set of females was used and every male was only tested once with each female. Females were size-matched (within 5% SL difference) and the two morphs randomly allocated to the small aquaria.

Male behavior was scored for 15 minutes from its first encounter with a female, defined as approaching a female to a distance below 20cm. Trials in which the male did not encounter both females and trials in which males did not court, were discarded from the analyses. Male behavioral patterns recorded were: neutral encounter, courtship displays (lateral display, dart, quiver, lead swim) and aggressive displays (frontal display and butting) (Baerends and Baerends-van Roon 1950). For the analyses, we chose the behavioral elements that can be always unequivocally attributed to courtship (darts) and aggression (butting) (MP, pers. obs.).

Gravidity stage was assessed by swelling of the female abdomen using the 5 point scale method of Seehausen and van Alphen (1998). Only ripening or fully ripe females (stages 3-5) were used in the experiments. After a trial, standard length (to the nearest 0.5mm) and weight (to the nearest 0.1g) were measured for each female.

Statistical analysis

Each experimental male was tested with six to eight different female pairs, and a weighted ANOVA with logit transformed proportion data (number of displays towards P females/total number of displays) was used to assess variation of preference between males.

In addition, we calculated repeatability for male mating preference (Lessells and Boag 1987).

To examine whether male mating preference (inferred from dart counts) could be predicted by mothers' genotype or by sibs' coloration, we fitted a generalized mixed effect model (GLMM) with a binomial distribution (Sokal and Rohlf 1995) and logit link function based on Laplace approximation (lme4 package in R; Bates and Sarkar 2006). Male identity was included as random effect to account for repeated measurements (Hedeker 2005). The significance of each term in the models was assessed using Wald statistics (Sokal and Rohlf 1995). We obtained the minimal model by exclusion of the non-significant two way interactions between terms. To assess the strength of the effects we calculated effect size *d* (Cohen 1988; Nakagawa 2004) and the respective confidence limits (Smithson 2003) where CIs including *d* indicate statistical significance.

Individual variation in male aggression behavior towards either female morph was tested as described for male preference. To identify a potential aggression bias towards one female color morph, we performed a GLMM model including male identity as a random factor and tested the overall intercept against zero.

RESULTS

Eighty-four preference choice tests on 12 experimental males were performed. Males courted one female morph, only, in 47.6% of the trials (see also Table 1). We found large individual variation in male mating preferences ($F = 5.80, p < 0.0001$, mean squares among males = 198, mean squares within males = 34; Figure 1). Repeatability for individual male mating preferences was $r = 0.39$.

The GLMM revealed that imprinting on sibs' coloration had no significant effect on male preference ($z = 1.42, p = 0.16$) nor was there any significant interaction between imprinting and male pedigree, i.e. mother's color morph ($z = 1.78, p = 0.08$). Male preferences were determined by the males' pedigree (minimum model including only male pedigree: $z = -2.96, p = 0.003$; effect size $d = 0.66$; CI: 0.21 – 1.11) (Figure 1).

Males displayed aggressive behavior in 39 trials and two males did not exhibit any aggressive behavior towards females (males BA2 and BB1, Table 1 and Figure 1). The overall intercept of the GLMM model was significantly different from zero ($z = 3.26, p = 0.001$) indicating an aggression bias towards P morph females. There was no significant effect of a male's pedigree on its aggressive behavior towards either female morph when using the minimal adequate model ($z = 1.47, p = 0.14$). The effect of imprinting on aggression bias was significant in the model including pedigree ($z = 2.42, p = 0.01$). This effect of imprinting was non-significant when the pedigree term was removed ($z = -1.72, p = 0.09$) and there was no significant interaction between imprinting and pedigree ($z = 1.70, p = 0.09$).

Pedigree (♀morph x ♂)	Rearing environment	Male	n trials	Darts per trial (mean ± SE)	single female courted
PxP	P	AA1	6	12.0 ± 2.13	0.83
		AA2	8	4.7 ± 1.47	0.86
		AA3	7	17.3 ± 5.47	0.29
	P+OB	AB1	6	13.7 ± 3.05	0.50
		AB2	8	7.0 ± 2.22	0.50
OBxP	P	BA1	8	14.2 ± 1.77	0.50
		BA2	8	15.2 ± 2.73	0.63
		BA3	6	3.2 ± 0.95	0.83
	P+OB	BB1	7	18.4 ± 1.70	0.00
		BB2	6	8.9 ± 2.23	0.14
		BB3	7	17.1 ± 2.28	0.00
		BB4	7	5.2 ± 3.01	0.80

Table 1 Treatment design and male IDs for the test of male preference mode of inheritance, number of trials per individual male, mean number of courtship displays (darts) per trial per individual (mean ± SE) and proportion of trials in which only one of the two female morphs was courted.

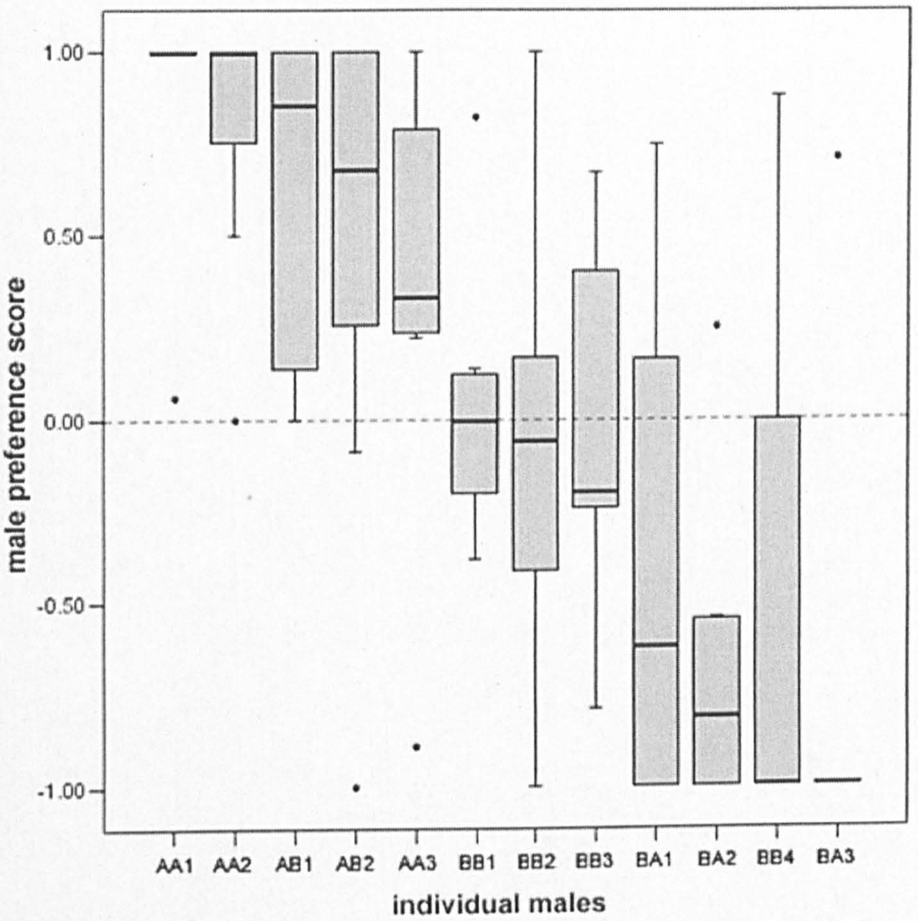

Figure 1 Individual male mating preference scores (dart displays to P female – dart displays to OB female/total number of dart displays). Positive preference score values indicate male preference for P females, negative values indicate male preference for OB females. Three-digit codes indicate male treatment group and identity, as given in Table 1.

Data are given as boxplot diagrams showing medians (middle line in the boxes), first and third quartiles (boxes) and range (whiskers). Outliers (◦) are also shown.

DISCUSSION

Female mate choice on male nuptial coloration is frequently cited as an important diversifying force in the extraordinary radiations of East African cichlids (e.g. Jordan et al. 2003; Pauers et al. 2004; Seehausen et al. 1997). However, so far little attention has been paid to the role male mate choice might play, although theoretical modeling has shown that under certain conditions male mating preferences could generate evolutionary dynamics leading to rapid speciation in sympatry (Lande et al. 2001; Kocher 2004).

Our results provide the first evidence of male mating preferences for female coloration in a polymorphic cichlid species from Lake Malawi. We show that the coexistence of mating preferences for alternative values of a trait in the other sex in a single population is not an unrealistic condition of models of sympatric speciation (Arnegard and Kondrashov 2004). It might not be coincidental that such variation in the direction and strength of consistent repeatable mating preferences for different female morphs occurs in a member of a rapidly speciating cichlid lineage.

Males from plain mothers (P) preferred to court plain females, males from blotched mothers (OB) exhibited either no preference or a preference for blotched females and male preference variation was best predicted by mother's color morph, suggesting genetic inheritance of the preference, with no apparent influence of rearing experience on male mating preference development. The individual variation in male preferences for female coloration measured in our experiment cannot be attributed to any maternal effect in the form of imprinting on mother's coloration, since we retrieved experimental males from their mother's mouth before natural release, excluding any possible visual contact between mother and offspring. Moreover, our preference trials design, precluding any non-visual communication between males and stimulus females, controls for any maternal influence in the form of imprinting on morph-specific non-visual (e.g. olfactory) cues that might have taken place during the mouthbrooding period.

That male preferences for female color morphs have evolved in the context of sexual selection is also suggested by the results of Knight and Turner (1999) on the species *P.* (*Maylandia*) 'zebra gold' (our study species), *P.* (*Maylandia) zebra*, and *P.* (*Maylandia) callainos*, all three comprising blotched female morphs and sympatric at Nkhata Bay, Lake Malawi. The authors explored the possibility that male species recognition might contribute to the observed complete reproductive isolation between these closely related sympatric taxa. Knight and Turner (1999) found that *P. zebra* and *P.* 'zebra gold' males were unable to distinguish conspecific from heterospecific females of either color morph, and courted both. Although individual variation in preferences was not considered in that study, these results indicate that male mating preferences in all three female polymorphic species play little role in species recognition.

The same female visual stimulus triggering male mate preference variation had a different effect on male aggressive behavior. Male aggression preferences for female color morphs appeared to be decoupled from male mating preferences. Males exhibited a general aggression bias towards the plain (P) morph, attacking more P than OB females. This general trend might be explained by negative frequency-dependent selection mediating aggressive behavior towards different color morphs. The P morph is the most common in all *P.* 'zebra gold' populations and OB males are extremely rare (Konings 2001). In the context of resource defense under predation risk, it might be adaptive to have a search image for the invading rival of the most common morph. This might also give a blotched (OB) morph a selective advantage in the early stages of invasion of a P population, since blotched individuals with the novel color patterns (blotch) would initially face less aggression from the predominant P morph (Mikami et al. 2004; Seehausen and Schluter 2004). Our experiment was not designed to examine differential aggression towards color morphs. Within-sex aggression experiments are needed to assess whether this pattern of aggression bias evolved in contexts other than sexual selection (e.g.

ecological competition) or is a secondary effect of intrasexual selection, in which males are selected to defend a spawning territory attacking males of the more common morph (P).

The female color and male preference polymorphism of *P.* *(Maylandia)* 'zebra gold' shows striking similarities with the *Neochromis omnicaeruleus* polymorphism studied by Seehausen et al. (1999) and considered as a case of arrested incipient speciation by selection on sex reversal and sexual selection. In both systems, variation in male mating preferences for either plain or blotched females is present. In *N. omnicaeruleus*, Seehausen et al. (1999) proposed that male mating preferences might have evolved by sex ratio selection to avoid matings with females carrying dominant female determiners in linkage with blotched coloration. Knight (1999) found evidence of dominant female determiners associated with blotch in our study species, *P.* 'zebra gold', and in its sister species *P. zebra.* Hence, similar forces might have shaped the evolution of male mating preferences in both the Lake Victoria cichlid *N. omnicaeruleus* and the Lake Malawi cichlid *P.* *(Maylandia)* 'zebra gold'.

Autosomal factors over-riding the effects of dominant female determining genes have been found both in *N. omnicaeruleus* (Seehausen et al. 1999) and in *P.* 'zebra gold' (Knight 1999). Males carrying such male-rescue factors are not affected by the sex ratio distortion deriving from mating with blotched females (i.e. carriers of dominant female determiners) Male mating preference variation might then originate by the possession or lack of male-rescue factors. There is preliminary evidence that blotched females are socially dominant over plain ones in *N. omnicaeruleus* and that this dominance is a pleiotropic effect of blotched coloration (P. Djikstra, pers. comm.). Social dominance might provide a selective advantage to blotched females e.g. in defending grazing grounds, such as patches of *aufwuchs*-covered rocks. Hence, individual variation in male mating preferences for female color morphs might be the expression of alternative tactics: males carrying male-rescue genes might be choosing superior (blotched) females without paying the costs of heavily biased offspring sex ratios. Males lacking rescue genes might avoid the costs of sex ratio distortion by mating with non-blotched females. We are now testing whether an association between male rescue genes and male mating preferences for blotched coloration is present in a wild population of males.

Finally, Lande et al. (2001) proposed an alternative explanation for the evolution of male mating preference variation in female color polymorphic species. The authors suggested that, when there is intense male-male competition for females and a pre-existing male preference for one color morph is present, males choosing 'unpreferred' females gain a selective advantage by incurring less competition for mating partners. If a male preference for non-blotched female coloration were found to be ancestral in *P.* zebra gold, as already shown for *N. omnicaeruleus* (Pierotti & Seehausen 2007), the origin of male preference polymorphism in this species might have been driven by the frequency-dependent selection envisaged by Lande et al. (2001).

We are only beginning to explore the complex interactions between natural and sexual selection in the 'blotch' sex-linked color polymorphism. Studies assessing the intensity of intra-sexual selection as well as the fitness value of blotched coloration in wild populations of *P.* 'zebra gold' are needed to shed light into the origin and maintenance of male mating preferences for female coloration in female polymorphic species.

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CHAPTER V

EVIDENCE FOR PARAPATRIC SPECIATION DRIVEN BY DIVERGENT MALE MATING PREFERENCES FOR SEX-LINKED FEMALE COLOURATION IN THE LAKE VICTORIA CICHLID FISH *PARALABIDOCHROMIS CHILOTES.*

ABSTRACT

The explosive radiations of cichlid fishes in the East African Lakes are often considered a prime example of diversification driven by sexual selection, however the role of sexual selection in speciation is still a highly debated issue and experimental work lags behind intense theoretical modelling.

Two adjacent island populations of the Lake Victoria cichlid *P. chilotes* differ markedly in female but not in male colouration. Using controlled lab conditions, allowing full interaction of individuals from both populations, we reveal extreme divergence in male mating preferences for female colouration and show that courtship is directed exclusively to females of own population. A microsatellite paternity analysis of clutches shows complete assortative mating between the two populations. A morphometric analysis, including these and other populations of the species, suggests that a scenario where sexual selection by male choice has been recruited by diverging natural selection differently acting on the two island populations, is unlikely. Preliminary microsatellite based population genetics data reveal low levels of gene flow between the two islands. We show that diverging male mating preferences have rapidly evolved between two adjacent populations of a Lake Victoria cichlid leading to full assortative mating in the absence of ecological diversification, suggesting rapid speciation by sexual selection on male preferences.

We discuss our findings with reference to the rapid explosive speciation of East African cichlids and provide a possible explanation for such unusual polymorphism in male preferences in the light of recent models of sympatric speciation by sex ratio selection and sexual selection.

Keywords

speciation, male preference, polymorphism, sexual selection, mate choice, *Paralabidochromis chilotes,* Lake Victoria, cichlid, assortative mating.

INTRODUCTION

It has long been recognised that geographic variation in sexually selected traits has the potential to drive reproductive isolation when trait and mate preferences co-vary between populations (Fisher, 1958; Lande, 1981; Kirkpatrick, 1982; West-Eberhard, 1983; Iwasa & Pomiankowski, 1995; Payne & Krakauer, 1997). Genetic drift, adaptation to different physical or social environments, pleiotropy, reinforcement after secondary contact and sexual selection are only some of the possible causes of divergence in both trait and preference (e.g. Wu, 1985; Schluter & Price, 1993; Howard, 1993; Qvarnstrom et al., 2006). There is now a large body of literature documenting variation in male sexually selected traits between populations (e.g. Wells & Henry 1992; Dale et al. 1999; Uy & Borgia, 2000; Tregenza et al. 2000; Masta & Maddison 2002). Geographic variation in female preference has received less attention (Butlin & Ritchie, 1991; Endler & Houde 1995; Ritchie, 1996; Jennions & Petrie, 1997; Simmons et al., 2001; Hebets & Maddison, 2005) and evidence of co-variation among populations of male sexually selected traits and female preferences is still relatively limited (Ryan & Wilczynski, 1988; Houde & Endler, 1990; Bisazza & Pilastro, 2000; Gray & Cade, 2000; Boughman, 2001).

Very little however is known about female trait variation between populations and its evolutionary consequences. Conspicuousness is far less common in females than in males (Andersson, 1994; Amundsen, 2000) although it has been suggested that research in this field has been hampered by misleading generalizations of empirical data as well as cultural stereotypic views on sex roles (Houde, 2001). Similarly, male mating preferences for female ornaments in non-sex role reversed species have been rarely documented (Amundsen & Forsgren, 2001; Bonduriansky, 2001; Houde, 2001; Wymann & Whiting, 2003; Weiss, 2006).

If male mating preferences for a female trait co-evolve with geographic variation in the trait, this might lead to assortative mating and possibly reproductive isolation between the diverging populations.

A female colour polymorphism common in both the Lake Victoria and Lake Malawi cichlid fish radiations provides an ideal system to test the hypothesis that divergence in female trait and male preferences for the trait between different populations can drive reproductive isolation in a parapatric or allopatric scenario.

In various species, females are either drab brown or yellowish with dark vertical bars (plain morph) or exhibit characteristic black blotches on a white, orange or yellow background (blotched morph). In the homozygote form of blotched colouration, individuals appear completely black or orange or white. Populations can be polymorphic for female colouration and interspecific crosses have revealed that some uniformly orange Lake Malawi cichlid species are in fact fixed for blotched colouration, since hybrids with non-blotched species exhibit black blotches (OS, AS, unpublished data). There is evidence that such colour polymorphism marks an underlying polymorphism in sex determining factors (Holzberg, 1978; Knight, 1999; Seehausen et al. 1999; Lande et al. 2001).

Male mating preferences for either female morph have been found in a polymorphic population of the Lake Victoria cichlid *Neochromis omnicaeruleus* (Seehausen et al. 1999; this thesis, chapter II) and similar individual variation in male preferences exists in the blotch polymorphic Lake Malawi cichlid *Pseudotropheus (Maylandia)* zebra gold (this thesis, chapter IV).

Recent modelling suggests that such colour and preference polymorphisms might be prone to rapid and repeated instances of speciation by the combined effects of selection on sex determination and mutual mating preferences (Lande et al. 2001; Pierotti & Seehausen, 2006; Vuilleumier et al. 2007).

To test whether geographic variation in female trait and male preference is responsible for reproductive isolation in parapatry or allopatry, ideally one would like to study populations in the act of diverging. Differences between species pairs that have long been reproductively isolated might not reflect the forces implicated in the speciation event but might have arisen well after isolation was completed.

Here we considered two adjacent island populations of the Lake Victoria cichlid *P. chilotes*, one fixed for plain (Makobe I. population) and one fixed for blotched female colouration (Ruti I. population). We tested in a semi-natural lab setting whether divergence in female colouration between these neighbouring island populations is associated with divergence in male mating preferences and whether this has resulted in patterns of non-random mating between the two populations. To exclude the possibility that the target of male mating preferences might be population-specific chemical cues, we subjected individual males to visual-only 2-way choice trials. Furthermore, we examined morphological variation between *P. chilotes* populations in order to identify possible local divergence in trophic ecology.

Finally, if divergence in female colouration and male preferences results in assortative mating, it should be possible to find a signature of restricted gene flow between the blotched (Ruti I.) and neighbouring populations. As a preliminary investigation in the potential structuring of populations driven by diverging mating preferences on female colour morphs, we examined the small available sample of *P. chilotes* from Makobe Island, Ruti Island and, in addition, the colour polymorphic population of Mabibi Island and a coastal site fixed for non-blotched colouration, i.e. Senga Point. Frequency-based and-individual based analysis of population structure for 4 *P. chilotes* populations were performed on multilocus microsatellite genotypes in order to explore potential patterns of genetic structuring between populations with different frequency distribution of female colour morphs.

MATERIALS AND METHODS

The species

Paralabidochromis chilotes, Boulenger 1911, is a haplochromine cichlid species endemic to Lake Victoria, East Africa. Males have blue to green flanks, an orange chest and blue dorsal fin, females can either be yellow-brownish (P morph) or black and white blotched (WB morph) (Seehausen, 1996). The species is non-blotched in most of its distribution but an island population of this species (Ruti Island) is fixed for blotch colouration and only recently (Aug. 2005) blotched individuals have been found in the Mabibi Island population.

P. chilotes is included in the IUCN RED LIST as *vulnerable*, i.e. "is facing a high risk of extinction in the wild in the medium-term future". Among the reasons justifying its placement in the IUCN Red List are the highly fragmented distribution of the species (Greenwood, 1959; Seehausen, 1996); slow growth and male sexual maturity attained later than in most other rockdwelling haplochromine cichlids (authors' pers. obs.), a contraction of the areas of occupancy and habitat decline in a highly specialised rock-dwelling insectivore, the introduction of invasive fish predators (e.g. the Nile perch) and the increasing pollution levels of the lake. Extensive long term work on the haplochromine cichlid community in Southern lake Victoria (Greenwood, 1959; Haplochromine Ecology Survey Team: van Oijen et al., 1981; Witte, 1981, Witte et al., 1992; Seehausen, 1996) suggests that *P. chilotes* is generally present with low population sizes at most sites.

Morphometrics

Male and female individuals from 4 populations (Makobe I., n=19; Ruti I., n=8; Mabibi I., n=8; Chamagati I., n=6) were measured with a digital calliper (to the nearest 0.5 mm) at 13 morphometric characters that have proven suitable to pinpoint even subtle shifts in feeding ecology in other cichlid species (Barel et al., 1977; Witte et al., 1997). Raw data were log transformed to obtain homogeneity of variance and to linearise allometric relationships. A multivariate analysis of covariance (with standard length as covariate) was performed on the transformed data.

A principal component analysis was run on the correlation matrix of the log-transformed morphometric data. Factor scores for the first principal component were retained for each individual and considered as a measure of size. A regression between log- transformed data and PC1 was performed using the pooled within-group slopes (Thorpe, 1976) and the residuals used in a discriminant function analysis (DFA) to identify the variable weightings maximising differences between populations.

2-way choice experiment

To test whether visual cues are sufficient to elicit male discrimination of females, four males from the P-population of Makobe Island (2 wild, 2 F1s), and three males from the WB-population of Ruti Island (2 wild, 1 F1), were simultaneously presented with a P female (Makobe) and a blotched WB female (Ruti), each confined in a PVC cylinder so that no chemical communication was possible between the experimental male and individual females. The number of male courtship and aggressive displays towards each female was scored for 15 minutes. A standardized mean preference score was calculated as the ratio (lateral displays to P female – lateral displays to WB female)/total number of lateral displays. A similar index was calculated for aggressive displays. Each experimental male was tested 2-3 times with different female pairs and the mean display and aggression scores were used in the analyses.

Free choice experiment

A partial partition design (Hert, 1989; 1991) was used: a 460x75x40cm aquarium was divided in five compartments by plastic grids allowing free movement of the females across the aquarium, whereas males, typically of larger size, remained confined to their individual compartments. These were fitted with bricks simulating a cave that acted as a refuge and spawning pit for the males.

The experimental aquarium was kept at 26 ± 2° C and illuminated with daylight fluorescent lighting on a 12:12h light dark cycle. The fish were fed twice a day with commercial flake food and a vitamin-enriched mix of mashed prawns and peas.

Four wild caught males, two from each population were assigned to individual compartments, avoiding males from the same population to be contiguous; the middle compartment was kept free for female refuges. Four females from each population, individually identified by the pattern of their colour markings, were introduced and then daily checked for spawnings.

A replicate was completed when a female from each population had spawned. This design is conservative as to the possibility of mate choice copying, the presence of which may only reduce estimates of assortative mating.

At the end of a replicate, one male from each population was then removed and replaced with a randomly selected wild male from the same population; positions in male compartments were also swapped so that, for each replicate, the same compartment was occupied by a different male from a different population.

During each replicate, 15-minutes point behavioural observations were performed for every male: sexual and aggressive interactions with individual females were scored. A replicate was discarded if a male was never observed being visited by females of each morph.

Paternity analysis

Before being introduced to the experimental tank, all test males and females were fin-clipped by cutting a small piece of tissue from the soft-rayed part of the tail and preserved in 100% ethanol for DNA-based paternity assignment. Brooding females were removed from the experiment after 5-7 days and clutches retrieved and preserved in 100% ethanol.

10% of the offspring from each brood (and at least 5 individuals, if brood size < 50) were typed at five (*Ppun5*, *Ppun7*, *Ppun17*, *Ppun21*, *Ppun32*) microsatellite loci, isolated from the closely related Victorian haplochromine *Pundamilia pundamilia/P. nyererei* complex (Taylor *et al.*, 2002). Preliminary testing on lab-bred individuals confirmed the suitability of these markers for paternity assignment in *P. chilotes*.

Population data

Microsatellite analysis

Samples were collected at 4 sites - the offshore islands of Makobe, Ruti and Mabibi and on the coastline at Senga Point - in the Southern Lake Victoria region, using monofilament gill nets. Total DNA was extracted from ethanol-preserved finclips with the HotSHOT protocol (Truett *et al.*, 2000). A total of 50 individuals were successfully amplified at 13 microsatellite loci: Ppun5, Ppun7, Ppun9, Ppun17, Ppun21, Ppun32 (Taylor *et al.*, 2002); Pzeb1, Pzeb2, Pzeb3, Pzeb4, Pzeb5 (Van Oppen *et al.*, 1997); Tmo-M5, Tmo-M11 (Zardoya *et al.*, 1996).

10µ l PCRs consisted of 1µl (ca. 20 ng) of template DNA, 1.0 µM each primer (one of which was FAM or HEX labelled), 200 µM of each dNTP, 0.50 units of Taq polymerase (Bioline), 1µl of 10x reaction buffer and 2.5 mM $MgCl_2$ (Bioline). PCR conditions for each locus were as follows: Ppun5: 3 min at 94°C, followed by five cycles of 94°C for 30s, 62°C for 30s and 72°C for 30s followed by thirty cycles of 94°C for 30s, 60°C for 30s and 72°C for 30s, followed by one cycle of 10 min of 72°C. Ppun7: 3 min at 94°C, followed by thirty cycles of 94°C for 30s, 57°C for 30s and 72°C for 30s, followed by one cycle of 10 min of 72°C. Ppun20 and Ppun21: 3 min at 94°C, followed by eight cycles of 94°C for 30s and 60°C dropping one degree every cycle (down to 53°C) for 30s and 72°C for 30s, followed by 25 cycles of 94°C for 30s, 53°C for 30s and 72°C for 30s, followed by one cycle of 10 min of 72°C.

PCR products were resolved by capillary electrophoresis on a CEQ8000 system (Beckman-Coulter) according to manufacturer recommendations, and fragments sizes were estimated with the fragment analysis module of the CEQ8000 Genetic Analysis System. MICROCHECKER 2.2.1 (van Oosterhout *et al.*, 2004) was used to identify possible genotyping errors in the sample due to stuttering, large allele dropouts or null alleles.

Statistical analysis of microsatellite population data

Frequency-based analysis

Genetic variability was estimated for each population as the number of alleles per locus (N_A), observed (H_o) and unbiased expected (H_E) heterozygosity (sensu Nei, 1978). These were computed with the program GENETIX 4.05 (Belkir et al., 2001). Allelic richness (A_R) and private allelic richness (A_P) were calculated with the rarefaction method of Kalinowski (2004) with the software HP-RARE 1.0 (Kalinowski, 2005).

Deviations from Hardy-Weinberg expectations for each locus and each population were calculated with the complete enumeration method (Louis & Dempster, 1987) for loci with less than five alleles, and using a test analogous to Fisher's exact tests (Guo & Thomson, 1992) estimated with a Markov Chain Monte Carlo series of permutations, for the other loci, as implemented in GENEPOP 3.3 (Raymond & Rousset, 1995).

Linkage disequilibrium was tested for all pairs of loci in each population and globally for each pair of loci across populations with Arlequin 3.01 (Excoffier et al., 2005). Critical significance levels for multiple testing were corrected following the sequential Bonferroni procedure (Rice, 1989).

F statistics according to Weir & Cockerham (1984) were calculated using GENETIX 4.05. The F_{ST} estimator θ was used, only, since many of our microsatellite loci are compound or imperfect and therefore do not fit the assumptions of the SMM (Balloux & Lugon-Moulin, 2002) required for Slatkin's (1995) R_{ST}. Moreover, F_{ST} estimators have been shown to be superior to R_{ST}-based analogues, when sample sizes and the number of loci available are small (Gaggiotti et al., 1999).

In order to visualize genetic relationships among populations, we used factorial correspondence analysis (FCA) to project individuals in microsatellite allele frequency space using GENETIX 4.05 (Belkir et al., 2001).

Individual-based analysis

We used a Bayesian model-based population assignment test as implemented in STRUCTURE 2.1 (Pritchard et al., 2000) to infer population structuring by estimating the number of populations with the highest posterior probability. An admixture model, in which individuals may have ancestry in more than one of the parental populations, was used. The posterior probabilities of the number of populations (K) were estimated without prior information on sampling location and using K (number of populations) = 1-6, with correlated frequencies. Markov Chain Monte Carlo simulations were run with 1000000 replicates and a burn-in of 500000 replicates for each K. The mean value of the resulting estimated Log probabilities from 15 runs at each K was taken and posterior probabilities of K were computed (Pritchard et al., 2000).

RESULTS

Morphometrics

The multivariate analysis of covariance on the ln-transformed measurements was highly significant (Wilks' λ, F = 89.165, $P < 0.0001$) and significant morphological differences between populations were found in body depth (F = 3.193, P = 0.035), lower jaw length (F = 4.205, P = 0.012), and snout width (F = 5.803, P = 0.002).

Makobe Island and Ruti Island individuals did not separate in DFA space although Chamagati Island individuals did separate from the other populations and Mabibi Island individuals only marginally overlapped in DFA space with Makobe I. individuals but not with Ruti Island individuals (Fig 1). A principal component analysis on the same data failed to separate individuals by population origin (Fig 2.).

2-way choice preference test

When olfactory communication was prevented, all seven males courted females from their own population, only, in all trials (binomial test, n=7, $P = 0.016$).

Makobe Island males and Ruti Island males did not differ in the mean number of courtship displays (Mann-Whitney: $U = 1.00$, $P = 0.114$)

Free choice experiment

Females visited all males' compartments and male courtship was observed in all trials.

Male courtship was observed in 28 instances. Males from the Makobe population and the Ruti population did not differ in courtship intensity (number of lateral displays: $t_{27} = -1.398$, $P = 0.174$).

Twenty-three broods were paternity tested. All clutches were the product of assortative matings, only. Brood size did not differ between Makobe and Ruti females: (t test: n = 23, $t_{21} = 0.658$, $P = 0.518$). No significant differences in spawning sequence with respect to population of origin were found (runs test: n = 23, $Z = 1.142$, $P = 0.253$)

Population structure

Expected (H_E) and observed (H_O) heterozygosities (Nei, 1978), total number of alleles (A_N), Allelic richness (A_R) and private alleles richness (AP) as estimated with the rarefaction method of Kalinowski, are presented in Tab.1, for three island populations of *P. chilotes* at 12 microsatellite loci.

Three loci (Pzeb1, Pzeb2, TMO-M11) significantly deviated from Hardy-Weinberg expectations in all four populations and were therefore excluded from further analyses. There was no evidence of linkage disequilibrium between any pair of loci ($P > 0.05$).

The pairwise θ_{ST} comparisons between populations suggested moderate population structuring, with all values significant at the 5% level (Tab.2).

The FCA plotting of the multilocus individual genotypes supported overall the clustering of individuals from different localities. In particular, the Makobe I. and the Ruti I. populations are distinct with no overlap in multilocus space, the Senga point population overlapping with all other three populations and Mabibi overlapping with Ruti I., only. The first three axes of the factorial correspondence analysis explained 14.17% of the total genetic variance.

The number of populations (K) with the highest posterior probability was found to be K = 2

The Bayesian analysis clustered together individuals from Makobe Island and Senga Point and suggested that the Ruti Island population consists of distinct multilocus genotypes, representing the second identified cluster, while the Mabibi Island population is composed of mixed ancestry with individuals being equally likely to be assigned to one cluster or the other (fig.4).

DISCUSSION

Geographic variation in mating preferences is the subject of growing interest as it can give us insights into which evolutionary forces drive preference evolution and its local variation and for the potential consequences for speciation. Two populations of the Lake Victoria cichlid *P. chilotes* have diverged both in female colouration and male mating preferences. Our experiment in semi-natural conditions, allowing full interaction between males and females of both populations, found patterns of paternity consistent with complete assortative mating and strict own population courtship behaviour, suggesting high levels of reproductive isolation between the Makobe Island and the Ruti Island populations of *P. chilotes*. Visual-only male choice tests confirmed that female colouration is sufficient to elicit population-specific male mating preferences in males of both Makobe and Ruti island populations, i.e. that no other population-specific cue, apart from female colouration, is required for male mate discrimination. This, together with the complete assortative mating observed in semi-natural setting (free choice experiment) confirm that male mating preferences for female colour patterns can drive pre-mating isolation between the non-blotched Makobe Island population and the blotched Ruti Island population of the cichlid *P. chilotes*.

If these results reflected natural patterns in the wild, we would expect to find a signature of restricted gene flow between Ruti Island and neighbouring populations of *P. chilotes*. At present, the available dataset allowed us only preliminary considerations that nevertheless are consistent with the conclusions deriving from the assortative mating experiment.

Our preliminary population genetic analysis outlined moderate but significant population differentiation between sites. The model-based Bayesian admixture analysis revealed the presence of two clusters, one corresponding to the blotched Ruti Island population and the second representing a large meta-population including all other sampled sites. There is no evident biogeographic reason for this result: indeed, Ruti Island is located between Makobe and Mabibi Islands and gene flow between all these islands is known to frequently occur for more stenotypic species (see also below). The pattern of population structuring suggested by the

Bayesian analysis is consistent with a scenario where alternative female colour morphs and male mating preferences largely prevent gene exchange between the Ruti Island population and the neighbouring non-blotched populations of *P.chilotes*. Mabibi Island, polymorphic for female colouration shows contributions from both the blotch-only Ruti Island population and non-blotched populations. However, caution should be applied in the interpretation of these very preliminary results. The main focus of our analysis is not the absolute value of genetic differentiation expressed by the F statistics (Weir & Cockerham 1984) between populations but rather possible signatures of asymmetric gene flow between populations polymorphic or fixed for alternative female colour morph patterns and male mating preferences. However, although accurate FST estimates are beyond the scope and interest of this study, caution is called also on the interpretation of the STRUCTURE results which, on the contrary, are more important for our hypothesis at test, i.e. whether populations fixed for alternative female colour morph and male mating preferences for female colour morphs generate patterns of restricted gene flow. While the small sample available for the population genetic pilot study represents an obvious limit, it is worth noting that it is consistent with our hypothesis. More work is needed to reach strong conclusions on this aspect of our investigation and, in particular, to exclude isolation by distance scenarios.

Previous experimental work has shown that behavioural isolation by female choice is a powerful force and a widespread phenomenon (e.g. Wells & Henry, 1992). Here we conclude that divergent male preferences on female colour patterns have driven substantial reproductive isolation between parapatric populations of a Lake Victoria cichlid.

What selective forces created and maintain strong assortative mating between Makobe Island and Ruti Island populations of *P. chilotes*?

The absence of significant divergence in morphospace between the Makobe I. and Ruti I. populations suggests that trophic local adaptation recruiting sexual selection favouring different ecomorphs at different sites is not a likely explanation.

There is no evidence that the visual predator community varies substantially in composition between Makobe Island and Ruti Island. Moreover, a blotched polymorphic species (*N. omni-caeruleus*) lives in sympatry with the non-blotched *P. chilotes* population at Makobe I. and non-blotched species are abundant with the sympatric blotched *P. chilotes* population at Ruti I., suggesting that predation pressure is unlikely to exert divergent selection on female colouration in the two islands.

We suggest that polymorphism in colour linked sex determining alleles might have generated male preference variation between Makobe and Ruti Island populations of *P. chilotes*. Blotched colouration is known to be physically linked to dominant female determining genes in all other colour polymorphic cichlid species examined so far (Holzberg, 1978; Seehausen et al. 1999; Lande et al. 2001; Knight, 2001; AS, OS, further unpublished data on Lake Victoria and Lake Malawi cichlid species).

In *P. chilotes* blotch colouration is limited to females and presents a heterozygote appearance (i.e. with black blotches on a yellow or white background, as opposed to totally black colouration characterizing individuals homozygote for blotch in other species). This suggests that, contrary to the standard male heterogamety assumed for most haplochromines, *P. chilotes* at Ruti island might be female heterogametic. Such reversals of heterogametic sex in species polymorphic for blotch and sex determination are in agreement with theoretical predictions (Bull, 1983; Lande et al. 2001).

As a consequence of matings between plain and blotched individuals, heavily female biased clutches result and Seehausen et al. (1999) suggested that male mating preferences variation for female colour morphs might have evolved to avoid such female bias effect, i.e. might have evolved by sex ratio selection.

We do not know whether 'blotch' is linked to dominant female genes in *P. chilotes* as appears to be the common pattern in all other blotch polymorphic species of lake Victoria and lake Malawi investigated so far. If confirmed, this might provide a cost for "hybrid" matings between Makobe and Ruti *P. chilotes*, since the 2 populations would have not only alternative female colour morphs and opposite male preferences but also opposite heterogametic sex. As a consequence, inter-population matings would generate female-biased progeny and possibly suffer clutch reduction due to the production of YY inviable offspring. This, in turn, would drive the evolution of male mating preferences against inter-morph matings. Similar evolution of male mating preferences to avoid the costs of sex biased progeny was observed in the Victorian cichlid *Neochromis omnicaeruleus* by Seehausen et al. (1999).

Although among-population variation in male nuptial colours appears to be no larger than the within-population variation (authors' pers. obs.), we cannot exclude the presence of subtle differences between Ruti and Makobe males that might enable females to discriminate and therefore play an active role in the patterns of assortative mating we observed in the present study. We argue that this is unlikely to be the case. In haplochromine cichlids, male courtship effort is a determinant factor for female mate choice. In fact, courtship ritual invariably consists in the male darting out its refuge and intercepting a cruising female crossing the male's territory. Repeated bouts of display are followed by attempts to lead the female to the male's refuge and spawning pit. Eventually, a female might respond by following the courting male to the spawning site, but, very frequently, the sequence is disrupted by the female fleeing. Therefore, females only approach and interact with courting males, i.e. a non-courting male will not be able to even interact with a female and therefore will have no chance of mating. We never observed a *P. chilotes* male courting a female of the opposite population in this experiment (or, in fact, in any other occasion): non-local females most likely did not consider such non-courting males as part of the mating pool. An implication is that experiments testing for female choice between local vs foreign males in *P. chilotes* would represent a challenge in experimental design since males would not court any non-local female and females would not consider any non-courting male. Video-playback might represent a solution although attempts by various research groups to elicit female response from video-playbacked courting males in cichlids have so far invariably failed, possibly because of the highly three-dimensional nature of male courtship.

For these reasons we conclude that female choice is unlikely to have contributed to assortative mating between Makobe and Ruti populations of *P. chilotes*, given the peculiarities of the sexual behaviour of haplochromine cichlids.

Future work is needed to reach a satisfactory characterization of the population structure of *P. chilotes* in Southern Lake Victoria. However, we anticipate that objective practical difficulties in collecting this elusive species and, most of all, conservation and ethical concerns on the long term survival of populations, will continue representing major difficulties.

In conclusion, we have provided the first evidence for male preferences on a female colour trait driving pre-mating isolation between parapatric populations. The evolutionary forces shaping such strong male preferences against inter-population matings might have to do with an unusual sex and colour polymorphism common in the explosive radiations of cichlid fishes in Lake Victoria and Lake Malawi. Finally, the fact that various species of Lake Malawi cichlids appear to be fixed for blotched colouration suggests that similar evolutionary pathways as the one described here for *P. chilotes* might have contributed to generate the extraordinary species diversity of cichlid fishes in the East African Lakes.

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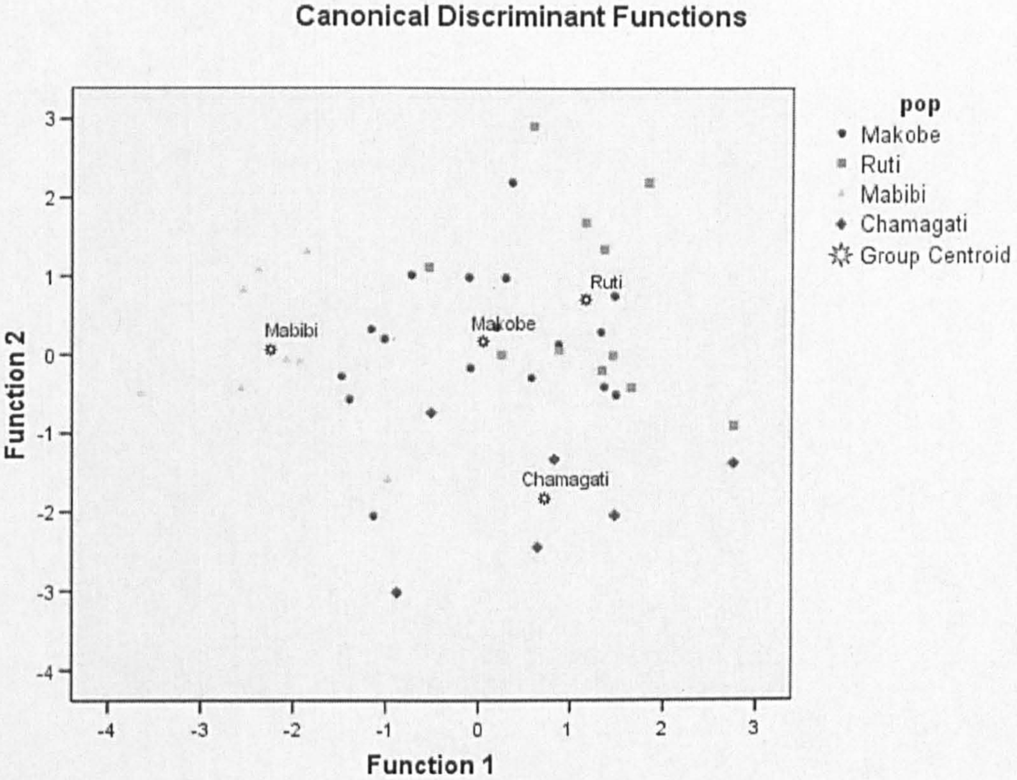

Figure 1 Discriminant function analysis on the residuals of the regression of the ln-transformed morphological traits on PC1.

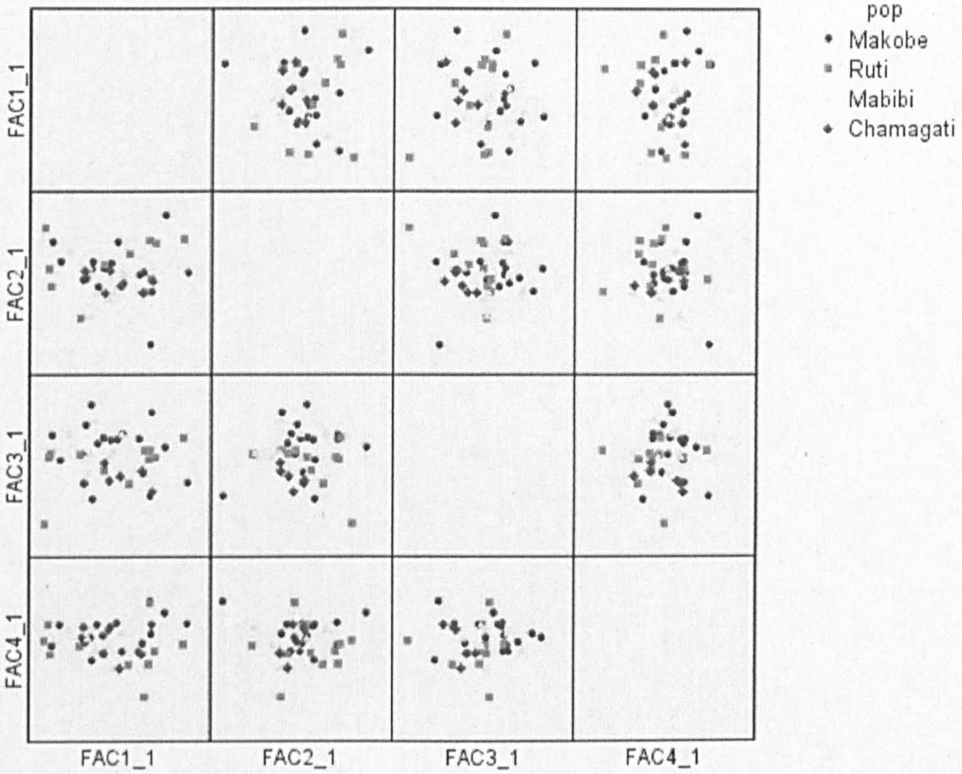

Figure 2 Principal component analysis on the same set of residuals as figure 1.

Population		Ppun5	Ppun7	Ppun17	Ppun21	Ppun32	Pzeb1	Pzeb2	Pzeb3	Pzeb4	Pzeb5	Tmo-M5	Tmo-M11	Average	Average including only Hw loci
Makobe Is.	H_E	0.9246	0.9122	0.7660	0.8734	0.6746	0.9604	0.8989	0.3689	0.3830	0.6250	0.9078	0.7872	0.76	0.72
(n = 24)	H_O	1.0000	0.8333	0.7917	0.9565	0.7500	0.7391	0.9167	0.3636	0.4167	0.4583	0.8333	0.5417	0.72	0.71
	A_N	15	14	12	18	3	26	15	3	2	3	14	14	11.6	9.33
	A_R	8.1683	7.8925	5.8956	7.4631	2.9848	9.8954	7.4001	2.2332	1.982	2.9491	7.6094	6.3283	5.90	5.24
	A_P	2.2758	3.7899	2.9257	4.6157	0.0006	9.1529	3.4525	0.2727	0.0001	0.0000	3.8146	4.3588	2.89	1.97
Ruti Is.	H_E	0.8644	0.7593	0.7593	0.8575	0.6958	0.9134	0.8851	0.4312	0.4046	0.6031	0.8968	0.8299	0.74	0.70
(n = 15)	H_O	0.9333	0.7143	0.7143	0.667	0.6429	0.7273	0.7333	0.4167	0.2667	0.3846	0.8571	0.4667	0.63	0.62
	A_N	10	9	6	8	4	11	10	2	2	4	14	9	7.42	6.56
	A_R	6.4992	5.7174	4.7004	6.2701	3.4147	7.9098	6.936	1.9977	1.9925	3.3163	7.9583	6.2224	5.24	4.65
	A_P	1.1402	2.4015	1.4032	3.0500	0.4289	6.5343	2.5357	0.0000	0.0000	0.4615	5.7608	3.1540	2.24	1.63
Mabibi Is.	H_E	0.8242	0.8132	0.8132	0.8791	0.6923	0.8636	0.7253	0.5275	0.4835	0.7121	0.6923	0.8571	0.74	0.72
(n = 6)	H_O	1.0000	1.0000	0.7143	0.8571	1.0000	0.5000	0.2857	0.2857	0.2857	0.3333	0.5714	0.8571	0.64	0.67
	A_N	5	6	7	7	4	6	5	2	3	3	4	7	4.92	4.56
	A_R	4.8462	5.5604	6.4066	6.5495	3.8462	6.000	4.7033	2.000	2.978	3.000	3.8462	6.5385	4.69	4.34
	A_P	0.8125	2.0228	2.4665	4.0075	1.0001	4.4511	0.4843	0.0001	0.9890	0.0055	1.0950	3.6536	1.75	1.38

Table 1 Unbiased (Nei, 1978) expected (H_E) and observed (H_O) heterozygosities, total number of alleles (A_N), for three island populations of *P. chilotes* at 12 microsatellite loci. Unbiased allelic richness (A_R) and private alleles richness (AP) as in Kalinowski (2005) are also shown.

	Ruti	Mabibi	Senga
Makobe	0.099*	0.109*	0.042*
Ruti		0.078*	0.083*
Mabibi			0.070*

Table 2 Population pairwise θ_{ST} values at 10 microsatellite loci. Significant values after Bonferroni correction are indicated with an asterisk.

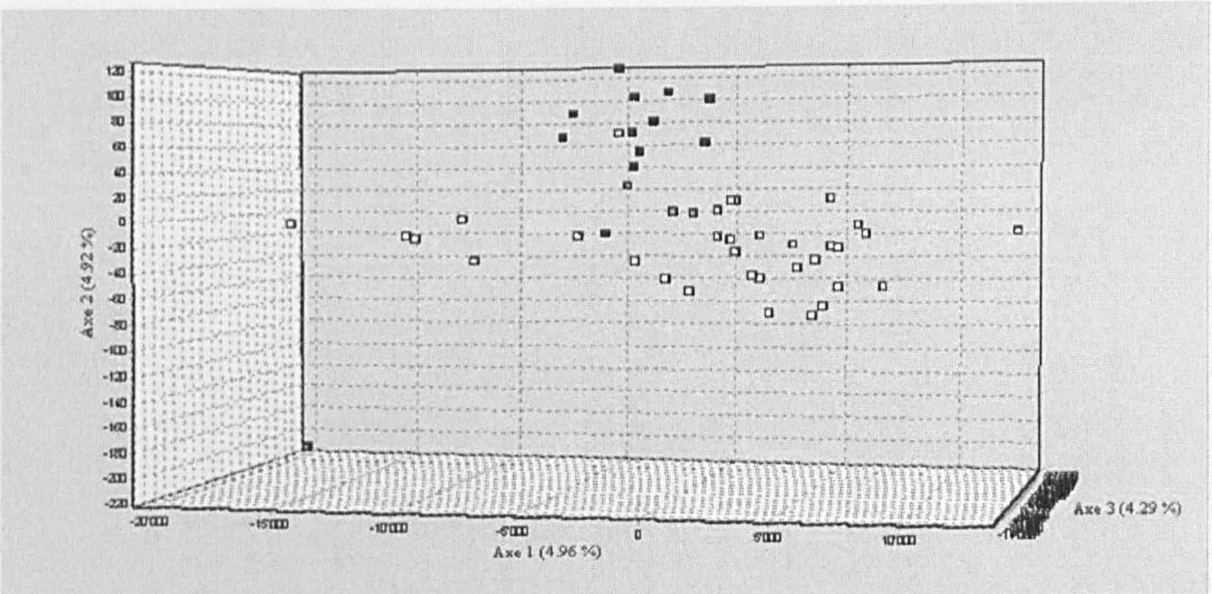

Figure 3 3D-Factorial correspondence analysis, as computed by Genetix, showing relationships among the multilocus genotypes of individual *P. chilotes*. Axis 1, 2, 3 are the first three factorial components. Individuals from Makobe Island are represented in yellow, from Ruti Island in blue, from Mabibi Island in white, and from Senga Point in grey.

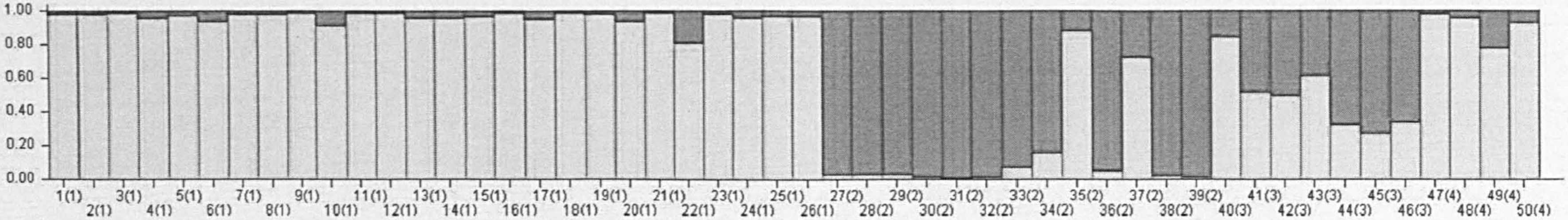

Figure 4 Estimated proportion of the coefficient of admixture of each individual's genome originated from population k, with k = 2. Each individual is represented by a column. Geographical origin of the samples is reported between parentheses (1. Makobe Island; 2. Ruti Island; 3. Mabibi Island; 4. Senga Point).

CHAPTER VI

UNRESOLVED ISSUES AND FUTURE WORK

Unresolved issues and future work

The main result from this thesis is probably the recognition that male mating preferences for indirect benefits can play a central role in the maintenance of colour polymorphisms and in driving population divergence. The association between sex determining genes and colour marker traits (as well as a suite of traits possibly pleiotropic to either sex or colour factors, e.g. aggressiveness) allows individuals to strategically allocate their reproductive resources and generate large individual variation in male mating preferences for female colour morphs. I have already stressed elsewhere that one of the biggest challenges in front of us now is the genetic characterisation of such preferences, the definition of the genetic architecture underlying their expression and, above all, a thorough analysis of the potential correlation of mating preferences between the sexes. Although Seehausen et al. (1999) suggest that such correlation is absent or at least that the expression of mating preferences in blotched females, if present, might be suppressed in this sex, no direct test has specifically addressed this issue. Lande et al.'s (2001) models of sympatric speciation do require mating preferences evolved in males to be expressed in the opposite sex. It has been suggested that there is selection against female mating preferences for blotched males, driven by differential mortality and consequent rarity of such males. This might have been achieved by female mating preferences determined by a different set of genes possibly sex-linked (P females do have strong preferences for P males) or by sex- or morph- limited expression. A detailed analysis of female preference distribution paralleling the work developed in chapter II on wild individuals is of paramount importance but a painstaking effort. Moreover, it would be very important to assess the female preference population as well as the male preference population at the same time since the two are most likely coevolving in cycles determining or being determined by fluctuations in relative morph frequencies.

The form of selection generated by male mating preferences is another field of great interest given that most theoretical analyses suggest that male mating preferences should be substantially different and exert very different selective pressures as compared to female mating preferences. Chenoweth & Blows (2003, 2005) provide possibly the only experimental analysis of mutual mating preferences and the different forms of selection generated by each sex on the population.

Pre-existing male mating preferences in Lande et al. (2001): for or against a novel colour?

The mechanism generating mating preference variation in Lande et al.'s models, i.e. a "minority advantage" effect for males mating with females rejected by the majority of the male population, relies on the pre-existing preference for local females (or against novel females). However, this pre-existing preference FOR local females might not be necessary: it would be interesting to model the situation where there is a pre-existing preference AGAINST local partners. Such a scenario is not unlikely when sensory bias sexual selection is involved. For example, swordtail fishes of the genus *Xiphophorus* exhibit a sexually selected elongated ventral caudal fin (the sword). Platyfish, which belong to the same genus, have swordless males, however females prefer conspecific males with artificial swords over normal, swordless ones (Basolo 1990). In other words, females of swordless species carry pre-existing mating preferences for males with swords even though such males are absent in their species.

If a population having a sensory bias for a certain trait that is absent from the population at present, is invaded by mutants with the novel trait, it is expected that most individuals will choose the mutants to mate. If most males choose, by sensory bias, novel females, the few males that stick to the local females lacking the sensory trap will suffer less male-male competition. Preference polymorphism might then be generated. If males had pre-existing suboptimal preferences before the invasion of the new female morph, preference variation would be not generated but rather maintained, this being possibly followed by increased divergence of the two preferences. Here the fact that sensory bias is driving the dynamics warrants that *most* individuals will express preferences for novel females and sustains the process for a sufficient amount of time. Moreover, sensory bias is generally thought to arise in contexts that are often unrelated to sexual selection, and for this reason it is likely to affect both sexes. This provides two advantages: first, the evolution of mate choice would only depend on the strength of sexual selection on the sexes, but the raw genetic material would be already in place available for selection to act. Second, it is more likely for a signal evolved in a non-sexual context by sensory bias to be non-costly, like the novel trait in Lande et al.'s models, which is neutral for natural selection on females.

Then, the assumption required for the generation of male mating preference polymorphism might become the existence of a male ancestral preference for the local females or an ancestral preference for the novel females.

This is of course highly speculative and it is not possible to make strong predictions without modelling the dynamics. However, it might be interesting to explore the parameter space in which Lande et al.'s (2001) mechanism for the generation of mate preference polymorphism can apply, given that the generation of sufficient variance in mating preferences represents a major difficulty in models of sympatric speciation by sexual selection.

One last observation on the evolution of mating preferences by the process envisaged by Lande et al.'s (2001). If male mate preference polymorphism can be generated by frequency dependent selection, once most of the population has a pre-existing male preference for one morph, it seems reasonable to consider whether this mechanism might apply to the evolution of preferences for multiple traits. It is immediate that the only way the necessary frequency-dependence might be generated is when the new trait interferes with the mate recognition system already in place. The simple addition of a new trait, without affecting the signal value of those traits already under sexual selection or their interaction in the mate recognition system, will not generate the necessary frequency-dependence on which Lande et al's mechanism is based. A possible exception might be represented by the presence of strong trade-offs between the pre-existing and the new trait so that evolutionary convergence on a single most attractive combination of traits cannot be achieved. Work on multiple sexually selected traits in guppies suggests that such trade-offs might not be uncommon (Brooks & Endler, 2001).

Blotch antagonistic? Some consequences and possibilities

Recent work has demonstrated intense exchange of genes between autosomes and sex chromosomes (Emerson et al. 2004). I suggested that reversals of the sex determining system should lead to large redistributions of genes with antagonistic effects between autosomes, and both new and old sex chromosome (here intended simply as the chromosome carrying the sex determining factor). When a dominant female determiner w on the X chromosome (W) invades, we might expect female advantageous male deleterious genes migrating to W. On the contrary, recessive X-linked male advantageous female deleterious genes would escape an X chromosome carrying w (W) which would never find itself in a male. In other words, while Y chromosomes have been accumulating in time male antagonistic genes, now W would be accumulating female antagonistic genes. As a consequence WY females might incur in deleterious effects due to the expression of previously male limited Y-linked genes in a female (WY are females unless they are also homozygote MM for the autosomal rescue gene M). Moreover, if the autosomal rescue M is slow in its increase in frequency, W might have the time to accumulate male deleterious genes and therefore WYMM males might incur in reduced fitness due to the expression of previously female limited W-linked antagonistic genes. This might provide an intrinsic potential disadvantage to WY males and contribute to explain why their frequency in the wild is lower than theoretical expectations. Maan et al. (*in review*) found that blotched males in the wild were at lower than expected frequencies already at very small sizes. This seems to contradict the hypothesis that blotched males are rare because of more intense predation by birds when engaging in courtship displays to females, as their frequency is already low when they are probably not yet sexually mature and holding a territory. An intrinsic fitness disadvantage might be consistent with these observations although there is no evidence at present in the lab (but no direct test of this hypothesis has been conducted yet). As an example, genes interfering with male gamete development are known to accumulate on W chromosomes in ZW/ZZ systems, and therefore might accumulate on the neo-W chromosome in *Neochromis omnicaeruleus*-like systems, too. As a consequence, WY males might have lower sperm quality than XY ones, this disadvantage preventing M from going to fixation. Such disadvantage might not be easy to detect with data from experimental crosses. If there is sperm competition in the wild, sperm precedence might explain blotched male inferiority without having any effect on fecundity *per se*, i.e. experimental crosses might not reveal any difference in clutch sizes between plain (XY) and blotched (WY) males.

The possibility that different male colour morphs might differ in sperm characteristics and/or competitive ability is an exciting area of future research, the rationale of which is represented by the different genotypes of male morphs, suggesting genomic conflict might be acting.

By the same token, WY females might be less fit than WX females, because of the accumulation of female detrimental genes on the Y. WY and WX sisters might be tested to identify differences in growth rate, condition, age at first clutch, mean clutch sizes, etc. Since WY and WX females are thought to be undistinguishable phenotypically, this test would be run initially without knowing the female genotype: all females from a cross expected to produce both WX and WY females would have to be individually identified and all parameters measured. When reached sexual maturity, test crosses with XY males would then be sufficient to infer female genotypes since only WX females would produce plain XX daughters.

The role of antagonistic genes might be important for a second reason. Lande et al.'s models are dependent on tight linkage between blotched colouration and sex determiner. However, Seehausen et al (1999) calculated a relatively high recombination rate ($r = 0.052$). This suggests that selection and not physical linkage maintain blotch and the dominant female determiner associated. Seehausen et al. (1999) proposes that selection on blotched males might explain the maintenance of the association between blotch and W, despite recombination.

If sex ratio selection plays a strong role in the system, there might be another source of selection to maintain blotch linked to the sex determiner. By advertising with the marker blotch whether they are carriers of a sex ratio distorter, females might elicit courtship only from males that are compatible with them, i.e. produce clutches with even sex ratios. Blotch would be maintained linked with w, since the identification of carriers of sex ratio distorters and the production of even offspring sex ratios is in the interest of both males and females. The honesty of the signal (i.e. linkage between colour and sex determiner) would then be maintained by sex ratio selection on both sexes. Females carrying the dominant female determiner w but lacking the marker trait blotch would be at a disadvantage since they would be courted by males lacking rescue genes eventually producing female biased clutches.

Finally, if blotch colouration was found to be sexually antagonistic, a third possibility for the maintenance of linkage between blotch and the sex determiner w, might be available. It has been suggested that blotched colouration in males is responsible for increased predation by birds (Seehausen et al. 1999; Maan et al. *in review*). There is also some preliminary evidence for pleiotropic effects of blotch on aggressive behaviour which might constitute a fitness advantage when defending feeding patches or in the establishment of dominance hierarchies (Dijkstra et al. in prep). Although the fitness value of blotch in males and females is still largely unknown, it is conceivable that blotch might have antagonistic female advantageous male deleterious effects. For example, it has been suggested that blotched colouration, although possibly associated to positive fitness effects such as dominance over plain individuals, might be detrimental to males because of their increased conspicuousness to predators during courtship displays. But here is the catch: a sexually antagonistic trait in linkage with the sex determining locus can reach relatively high frequencies even when at large distances from the sex determining region as shown by Rice (1987). How tight linkage has to be to support the accumulation of a sex-linked antagonistic trait will depend on the amount of dominance and the size of the beneficial effects on the favoured sex. Complete dominance of blotch in *Neochromis omnicaeruleus* has been established by the experimental work of Seehausen et al. (1999), setting favourable conditions for our hypothesis. However, without a detailed quantification of the fitness effects of blotch in both sexes, we cannot draw any strong conclusions yet. The possibility that linkage between blotch and the dominant female determiner is maintained by sexually antagonistic selection allowing relatively large recombinational distances between the two loci, is a new hypothesis that might help reconcile some aspects of the modelling with data from *N. omnicaeruleus*. Accurate estimates of fitness of blotch in both sexes might prove to be challenging for two reasons. First, as new data on blotch polymorphisms in different species accumulate, it becomes more and more evident that blotch is likely to affect fitness in many different contexts, from predation to social dominance, to other yet unexplored potential pleiotropic effects e.g. on immune defences, given melanin's antioxidant activity as well as its key role in immunocompetence (Rantala et al. 2000) and its susceptibility to UV light and consequent melanoma formation (Setlow et al. 1993). This adds up to the inherent difficulties in measuring fitness components in natural populations.

...and if blotch is antagonistic: alternative pathways.

Lande et al. (2001)'s sequence of evolutionary events leading to the evolution of variation in sex determining factors, W-linked phenotypic markers and male and female mating preferences, begins with the invasion of a new dominant female determiner. Only after W is established in the population, a W-linked trait appears. The authors point out that a colour trait can only appear on W as this is the only way it would not be selected against by female choice.

Other scenarios including sex linkage of a trait and invasion of dominant female determiners are possible. Here I suggest one, the invasion of a sexually antagonistic colour gene in a male heterogametic species, followed by the invasion of a new sex factor (i.e. the opposite sequence of events of Lande et al. (2001)).

Under the untested assumption that blotch represents a female antagonistic gene, let's imagine the invasion of a dominant female antagonistic gene (the colour trait blotch) in a XX/XY system. A dominant mutation CAN reside on an X chromosome as long as it is more advantageous to females than to males. In fact, the mutant gene will spend 2/3 of its time in females and as a consequence it will be positively selected even if heavily deleterious to males. After the establishment of blotch on the X chromosome, a dominant mutation on X, changing genotypic males (XY) into females (the dominant female determiner W of Lande et al. 2001) will be favoured because it leads to the resolution of the sexual conflict generated by the antagonistic gene (blotch). In fact, during the invasion of W, W'X', W'Y, X'Y and YY individuals are produced. Selection on males assists the reversal from male to female heterogamety by favouring YY individuals over X'Y, i.e. the invasion of W is positively selected because it generates (via the production of W'Y females) males free from the antagonistic effects of blotch (YY males). Sex reversal changes the expression of blotch from a positively selected genomic location (on the X: female-biased expression) to an even more favourable location (on the W: female-limited expression).

The evolution of mating preference variation might be achieved in various ways, under the verbal model proposed here. What follows is just one of the possible pathways and in the case I propose here, it is mainly driven by female preferences and not male preferences as in Lande et al. (2001).

After the invasion, blotch reaches intermediate equilibrium frequencies, i.e. the female population will be polymorphic for the antagonistic mutation. Females regardless of whether they carry the antagonistic gene, will develop preferences for male carriers over non-carriers, since this leads to the most favourable net offspring fitness. If preferences are not sex limited in expression, males might inherit mating preferences for the antagonistic trait (blotch) from their mother and choose females of their mother's colour. Males might choose blotched females even in the absence of pleiotropy between the sexes, since they would receive the fitness advantage to their daughters, and the fitness cost suffered by their sons would be lessened by the fact that sons would inherit only one of their mother's X chromosome (e.g. Albert & Otto 2005). The antagonistic gene and mutual mating preferences for it might then spread in this population. Rice (1987) modelled the invasion of a dominant female advantageous trait on the X chromosome and calculated equilibrium frequencies as a function of the degree of dominance and the fitness advantage to females and disadvantage to males. But it is yet to be explored how mutual mating preferences for the antagonistic trait might affect the equilibrium frequencies and particularly whether directional sexual selection in both sexes for the antagonistic trait could push it to high frequencies and possibly fixation.

At this stage in the process, a population has been invaded by an antagonistic gene and mutual preferences have evolved for this trait, favouring its increase in frequency. If we introduced Lande et al.'s condition of an ancestral male and female mate preference, we would probably obtain some level of premating isolation between an "ancestral population" with ancestral mutual preferences and the population invaded by the antagonistic gene and with mutual preferences for it.

But I will not use the assumption of pre-existing preferences against blotch, since the action of sexual antagonism, first, and sex reversal, later, is sufficient to generate divergent female mating preferences. So far, from a population that has no preferences, we have generated mating preferences for a female antagonistic gene in a population. The invasion of a female sex reversal gene in a X'X'/X'Y population carrying the antagonistic gene, as I suggested above, might be favoured by selection as it generates females carrying the female advantageous antagonistic trait and males free from the trait (YY). A new female mating preference for non-carrier males (YY) might evolve since YY males have higher fitness than males that are carriers of the antagonistic gene. Eventually, a female heterogametic population with blotched females and non-blotched males emerges: males exhibit preferences for blotch females and females have preferences for non-blotched males. This population might be isolated by female mate preferences (and opposite heterogamety) from a population not invaded by the sex reversal gene.

In conclusion, the suggested dynamics is very simple: a dominant X-linked female antagonistic trait invades a XX/XY population, as predicted by theory (Rice 1987). Female mating preferences evolve for the trait in males. The invasion of a female sex reversal gene is favoured as it restricts the antagonistic gene to females only, resolving sexual conflict. New female preferences for newly generated antagonistic gene-free males, appear. Eventually, a population with female heterogamety and mutual mating preferences emerges.

The interest in this verbal model lies in the fact that i. the antagonistic characteristics of the trait directly explain both the genomic location of the trait at any point in the model as well as the generation of mating preferences from a population with potentially no preferences; ii. a sex reversal gene is favoured because it leads to the resolution of sexual conflict. iii. the verbal model does not require pre-existing preferences in the population: on the contrary, mating preferences for and against the antagonistic trait evolve as a direct consequence (good genes) of the antagonistic properties of the trait; iv. the verbal model can be driven by female choice only. Hardly the *N. omnicaeruleus* system can be fitted in such model, unless male preferences are the expression of preference genes selected in females. But it is interesting to consider other ways in which sex determining factors and genes with antagonistic effect might co-evolve and possibly generate divergence, under assumptions that, in this case, are less restrictive than Lande et al.'s (2001).

The Neochromis W as a driving sex chromosome?

If YY are inviable, W behaves as a selfish genetic element, being transmitted to two thirds of the offspring, i.e. W might "drive". However the potential advantage deriving by the transmission ratio distortion, is counteracted by the decrease in fertility due to the loss of ¼ of the offspring

(YY individuals), unless a mechanism of compensation is in place, as observed in some lemming species.

It is unclear however, how such compensation might take place in *Neochromis omnicaeruleus* and other species polymorphic for dominant female determiners. In haplochromine cichlids, maternal care is provided by mouthbrooding the eggs until the fry have consumed the yolk and become free swimming: within a cycle of egg maturation, if a time-window is available to females to release successive bouts of eggs, YY unviable individuals might be expelled from the mouth and the female could fertilise additional eggs with a different male. There is evidence of multiple mating in Lake Malawi cichlids and interestingly in one species polymorphic for a blotch-linked dominant female determiner (Kellogg et al. 1995; Knight et al. 1998). Selection for multiple mating might be more intense on blotched females. In fact, while plain XX females are not affected by the problem of producing unviable YY individuals and they never produce female-biased clutches regardless of the male they mate with, blotched WY females will inevitably suffer a fecundity disadvantage (unless other forms of compensation are present). Such mechanism however would be highly imperfect because multiple mating would only asymptotically increase the number of viable offspring but full compensation would never be achieved. Alternatively, a mechanism of selective maturation of only W-bearing eggs, as in the varying lemming *Dycrostonyx* ssp., might achieve full compensation. In that case, W would represent an efficient driving sex chromosome. This last hypothesis is undoubtedly very exciting and could be easily tested in the future, by first creating WY individuals from crosses between WB and OB females. The only heterozygote blotched females generated would be WY. Next, WY females could be mated with plain males and the offspring examined for their genotype by test-crosses. The prediction is that WY females would contribute only W gametes to the offspring, hence in crosses with plain (i.e. XY) males, only WX and WY individuals will be produced. But if WY females regularly produce both W and Y gametes, plain sons XY will appear in their clutches. Full compensation could be deduced from presence/absence of plain males in crosses between WY females and XY males.

If compensation is achieved by other mechanisms or is only partial (i.e. some YY inviable are produced), its detection might be more challenging and possibly achieved by comparing multiple clutches of WX and WY sisters with a common set of males, and analysing differences in clutch size. A standard quantitative genetics approach would be probably required, a difficult challenge given the relatively long maturation period of haplochromine cichlids, strong mating preferences in males and females as well as the failure so far of artificial fertilization attempts.

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