Early learning and speciation : the effects of early experience on sexual and aggressive behaviour in Lake Victoria cichlid fish

Verzijden, M.N.

Citation

Verzijden, M. N. (2008, January 9). *Early learning and speciation : the effects of early experience on sexual and aggressive behaviour in Lake Victoria cichlid fish*. Retrieved from https://hdl.handle.net/1887/12549

Chapter 4

Cross-fostering does not influence the mate preferences and territorial behaviour of male Lake Victoria cichlid fish.

Machteld N. Verzijden, Juul Zwinkels, Carel ten Cate

Abstract

The ability to recognise conspecifics in contexts of mate choice and territorial defence may have large effects on an individual's fitness. Understanding the development of assortative behaviour may shed light on how species assortative behaviour evolves and how it may influence reproductive isolation. This is not only the case for female mate preferences, but also for male mate preferences and male territorial behaviour. Here we test with a cross-fostering experiment whether early learning influences male mate preferences and male – male aggression biases in two closely related, sympatrically occurring cichlid species *Pundamilia pundamilia* and *P. nyererei* from Lake Victoria. Males that had been fostered, either by a conspecific female or a heterospecific female, were tested for their aggression bias, as well as for their mate preferences, in two-way choice tests. Males cross-fostered with conspecific and heterospecific foster mothers selectively directed their aggression towards conspecific intruders. The cross-fostering treatment also did not affect male mate preferences. These results are in striking contrast with the finding that females show a sexual preference for males of the foster species.

Key Words: Aggression, fish, male mate choice, *Pundamilia pundamilia*, *Pundamilia nyererei*, reproductive isolation, rival imprinting, speciation, sexual imprinting, territoriality.

Introduction

Understanding how new species evolve and persist is a key issue in evolutionary biology. One of the more controversial issues is how this may happen while the (incipient) species co-occur. This process of sympatric speciation has long been thought of as improbable, but recent empirical work has revealed several possible cases (e.g. Bush 1969; Schluter & McPhail 1992; Seehausen & van Alphen 1999; Kornfield & Smith 2000; Korol et al. 2006). Sympatric speciation might occur under a number of critical conditions, and the developmental mechanisms for mating preferences and aggression biases may have direct bearing on a number of them. First, reproductive isolation should arise very quickly between the diverging phenotypes (reviewed in Kirkpatrick & Ravigne 2002). This means that individuals should be mating assortatively, mediated by their mating preferences. If both sexes have assortative mate preferences, this could strengthen the assortative mating pattern (Almeida & de Abreu 2003; van Doorn et al. 2004). Mutual mate choice is commonly found in species where both sexes substantially invest in the offspring (i.e. Amundsen 2000). Although male mate preferences are not a priori predicted in polygynous mating systems it is found in several polygynous (review in Amundsen 2000), and even in lekking bird and fish species (Saether et al. 2001; Werner & Lotem 2003; Pierotti & Seehausen 2007). It may thus occur more often than previously thought. Mating preferences are assortative if individuals prefer mates with a similar phenotype as themselves. Therefore, the critical question is how mating preferences arise. Especially in a population with rapidly changing phenotypes, the extent of assortative mating may be quite different between different developmental mechanisms (Kirkpatrick 1982; Laland 1994; ten Cate 2000, this thesis chapter 3).

A second critical condition is that the coexistence of both incipient species can occur. This may be possible under negative frequency dependent selection (e.g. reviewed in van Doorn et al. 2004; Rueffler et al. 2006). Such selection promotes invasion of a new phenotype into the population, and at the same time allows stable coexistence of two phenotypes. Male-male aggression may yield such selection when males direct their aggression strictly against males of a similar phenotype, giving males of a rare phenotype the advantage of having fewer aggressive interactions (Mikami et al. 2004; Seehausen & Schluter 2004; van Doorn et al. 2004). This may for instance occur when males that compete for the same females show more interest in chasing each other away from their mating grounds than in chasing males competing for different females. Because aggressive interactions are likely to be costly, selective aggressive behaviour has a direct bearing on the fitness of an individual. Therefore, aggression biases towards males that compete for the same females or the same resources (i.e. conspecific males), may be adaptive in the sense that this avoids unnecessary competitive interactions. Aggression is often found to be directed more at conspecifics. In birds, for instance, males respond more to conspecific song than to sympatric heterospecific song (reviews in Becker 1982; Irwin & Price 1999), túngara frogs respond indiscriminate to allopatric heterospecific calls (Bernal et al. 2007), and in fish males also respond more to conspecific territorial intruders (Genner et al. 1999b; Dijkstra et al. 2006b). As for mate preferences, the question is how the direction of aggression develops, because this critically influences the degree of assortative aggression.

Because of its central role, the study of developmental mechanisms for species recognition will improve our understanding of how the degree of assortative behaviour (mating, aggression) changes with new circumstances, and how assortative mating patterns can arise. It could ultimately also give us insight in how this behaviour influences speciation processes.

One mechanism that may promote assortative mating particularly is sexual imprinting (Laland 1994; Irwin & Price 1999; ten Cate 2000, this thesis chapter 2). Sexual imprinting is a form of learning, in which young animals learn about the phenotype of an individual, usually a parent, and use this as a model for future sexual preferences. It is a well documented phenomenon, especially in birds (reviewed in ten Cate & Vos 1999), but also in other taxa (Kendrick et al. 1998; Bereczkei et al. 2004, this thesis chapter 3), and has recently gained attention in the context of reproductive isolation. Sexual imprinting may aid the evolution of assortative behaviour in two ways. First because it immediately provides preferences for newly arising phenotypes (ten Cate & Vos 1999; Irwin & Price 1999) and, second, because it ensures a strong link between the phenotype of the individual and its species assortative behaviour (Laland 1994; ten Cate & Vos 1999; Albert 2005, this thesis chapter 2).

Mate preferences might also develop by learning at a later stage in life through (sub) adult experience (e.g. Dugatkin & Godin 1992; Schlupp & Ryan 1997; Hebets 2003). Alternatively, different mating preferences may be predominantly determined by genetic differences (e.g. Shaw 2000; Ritchie 2000). All these developmental mechanisms are not entirely mutually exclusive. They vary however in the degree of assortative behaviour they may mediate. Learning behaviour at a later stage in life may not produce preferences for phenotypes similar to an individual’s own phenotype. This is because learning will often take place through experience with individuals that are relatively unrelated, which hence may not share the same phenotype or genotype. When genetic differences account for the developmental differences in preferences, these will in general not be very plastic. This means that in populations with quickly evolving phenotypes, preferences may lag behind and hence will not be assortative.

Imprinting might also link the direction of aggression towards males of a similar phenotype as themselves. Two studies on bird species demonstrated such imprinting, (Vos 1994; Hansen & Slagsvold 2003), coined ‘rival imprinting’ by Hansen & Slagsvold. However, a central American cichlid showed no evidence of such imprinting (Barlow & Siri 1987).

It is possible that there is a relation between a male’s mate preference and his aggression bias. Such a bias may be internally coupled, for instance through a genetic linkage, when males’ aggression biases and mate preferences are both genetically determined. It may also be that learning in one context, for instance learning which male is a rival, has consequences for a males' mate choice. Experience in one context, indirectly influences a male's mate preferences. Such a coupling of behavioural biases would then be genetically determined, but the biases themselves may not have to be. Finally, unrelated experiences in aggressive and mate choice contexts may also yield high degrees of assortative behaviour in both contexts, and thus a relation in the behaviour between the two contexts.

Case studies for sympatric speciation are the East African cichlid species flocks. East African lakes harbour up to 1100 endemic cichlid species (conservative estimate, Turner et al. 2001). The observation that many sympatric species pairs differ in male nuptial coloration, but are morphologically very similar, has fostered the hypothesis that sexual selection was a major driving force in their speciation (Seehausen & van Alphen 1999; Kornfield & Smith 2000). Male cichlids defend territories vigorously against other males in a lek-like aggregation in order to attract and court females and eventually mate with them (Maan et al. 2004; Seehausen & Schluter 2004). The sympatric species pair *Pundamilia pundamilia* and *P. nyererei* is studied as a model for speciation research in Lake Victoria cichlids. Males of these species, originating from wild populations, are known to show more aggression towards a conspecific intruder than to a male intruder of the sister species (Dijkstra et al. 2006b). Also, female mate choice was found to be assortative with regard to species identity (Seehausen 1997; Seehausen & van Alphen 1998).

In a previous paper, we demonstrated evidence that female mate preferences are mediated by sexual imprinting on their mother's phenotype (chapter 3). In this study, we focus on the mechanisms that mediate the male-male aggression biases and male mate preferences in these species. Males of both *P. nyererei* and *P. pundamilia*, were raised in an interspecific cross-fostering experiment, after which each male was tested for its species assortative behaviour in two contexts, territorial defence and mate choice. With these experiments we tested the following questions. First, is male territorial defence mediated by imprinting on the maternal phenotype, like that of the females? Second, are male mate preferences, if there are any, mediated by sexual imprinting? We examine the degree of assortative behaviour of the males in the two different contexts, and we also compare the results for male mate choice with those for females, as presented earlier (chapter 3). The implication of the results for our understanding of speciation in these species is discussed.

Methods

Housing and Cross-Fostering of the Animals

Wild caught *P. pundamilia* and *P. nyererei* from Makobe island, Tanzania (Seehausen & Bouton 1997), were housed in single species stock tanks (size 1 x 0.4 x 0.6 m). For breeding, up to 12 females were housed with one male that was replaced regularly. All tanks were connected to a central recirculation water filter system. Water temperature was 24.5 +/- 1° C; the light regime was a 12 :12 h light:dark cycle. Fish were fed daily with fresh shrimp and peas or commercial pellets and flakes. Brooding females that had spawned approximately at the same time (maximum 4 days apart), were gently forced to spit out their eggs, within 2-5 days after spawning. Eggs were then taken up in a plastic pipette, which was then emptied in another females' mouth, such that each female received the eggs of the other female. All four types of egg exchanges were done: both within and between species. Each treated female was placed in a visually isolated small tank (25x25x10 cm). Mouth brooding takes 3-4 weeks, then the female releases the fry. Fry then start foraging independently, while females guard them during 3 weeks. This occasionally stopped prematurely, in which case the female was placed behind a perforated transparent sheet within the tank, maintaining both visual and olfactory communication. Females were removed after 4 weeks. At 1.5 to 5 months after spawning, each brood was placed in a stock tank exclusive for that brood. With first signs of nuptial coloration (average 170 days), we visually separated brothers and sisters with a perforated opaque sheet, to prevent them from gaining breeding experience. At sexual maturity, PIT tags (12 mm glass tags, UKID122GL Biomark Inc., Idaho, USA) were implanted in the left belly cavity. Males were then placed in a communal tank with conspecific males of other treatment broods. Each male was first tested twice for aggression bias towards each of the two species, and then twice for mate preference, so that each male was tested four times. The inter-test interval was at least one week. All tests were done blindly with respect to the foster-treatment of the male. The wild caught parental generation was kept in our facilities for further breeding.

Male Behaviour

Adult males defend territories in order to attract females. Territories in these species are only for reproduction, feeding occurs outside the territories (Seehausen & Schluter 2004). Aggressive interactions usually take place at the border of the territories, to repel intruders. We recorded four types of behavioural displays during aggressive interactions (Baerends & Baerends-van Roon 1950; Dijkstra et al. 2006b): Frontal displays, bites, lateral displays and quivers. During frontal displays, males line up head to head. During bites, males grab each other's mouth and pull back and forth. This is usually preceded by a frontal display. In our set-up males could not physically bite each other, but they bit the Plexiglas separating them (see below). We added the number of times for frontal displays and bites into one measure (abbreviated with FD-B), as some males never performed bites and some never performed frontal displays prior to attacking, while others performed both. During a lateral display, the male extends his dorsal, anal and pelvic fins, and positions itself such that its flank is in front of the head of the opponent. The quiver, finally, is usually preceded by a lateral display. The male has the same position as in the lateral display, and the fins are equally extended, but a fast shaking movement of the body is made. Lateral displays and quivers are also sequentially connected behaviours, and again, some males never performed quivers or lateral displays. We therefore took lateral displays and quivers as one measure also (abbreviation: LD-Q).

Lateral displays and quivers are also used in courtship (Baerends & Baerends-van Roon 1950; Seehausen & van Alphen 1998). The difference in the displays between the two contexts is that during aggressive interactions, the operculi are opened and the lower jaw is held at a wider angle. This gives the appearance of a broader head. During courtship, the approach of a male to a female may start a sequence of displays, starting with a lateral display, followed by a quiver and a lead swim and potentially ending in spawning. Females may respond to these behaviours by approaching the male and following him to the centre of his territory, although in our setup females could not fully complete the follow to a lead swim (see below). We scored the number of lateral displays, quivers and lead swims in the mate preference tests. We analysed male courtship behaviour per display sequence. We also noted the time the focal individual spent with each male or female. In both experiments, we used Observer 3.0 (Noldus information technology, Wageningen, The Netherlands).

Male Aggression Tests

The focal male was placed in the experimental tank (1x0.4x0.5 m) 48 hours prior to testing. Each male also had a 'neighbour male': a smaller male of a different Lake Victoria cichlid genus placed at one end of the tank behind a Plexiglas sheet. We placed this male in the experimental tank to enhance territoriality (Dijkstra et al. 2006b). At the time of testing, two cylinders were placed in the experimental space of the focal male. One stimulus male was in each cylinder, making up a stimulus pair, with one male of each species. Stimulus males were matched in standard length (mean difference +/- SE = 0.4 +/- 0.1 mm). Observations started right after placement of the cylinders, and lasted until at least 5 minutes of aggressive interactions had passed. Each male was tested twice, and male intruder species position was reversed in the second test. We tested 7 *P. pundamilia* control males, 15 *P. pundamilia* cross-fostered males, 6 *P. nyererei* control males and 12 *P. nyererei* cross-fostered males, from in total 19 broods. In total we performed 80 aggression tests. As stimulus males we used 26 *P.pundamilia* males and 29 *P.nyererei* males, which were combined to 35 stimulus pairs. A focal male never encountered the same stimuli in the second test.

Male Mate Choice Tests

The focal male was placed in the experimental tank (2x0.4x0.5) 24 hours prior to testing. Also, at both ends of the tank, a Plexiglas sheet was placed, behind which a smaller male from a different genus was placed. This was again done to enhance territorial behaviour in the focal male. The reason for using two males in this case was to prevent side preferences, which might arise from the larger experimental space. We placed two 6-sided Plexiglas cylinders with 5 mm holes at equal distance from the centre of the tank. Water flow was directed into each of the cylinders. We placed one female into each cylinder, and the two females formed a stimulus pair, with one female of each species. Before the male was placed in the experimental tank, 6-sided opaque PVC cylinders were placed around the slightly smaller Plexiglas cylinders, to hide the females from the males until the time of testing (24 hours later). We used 43 *P. pundamilia* and 34 *P. nyererei* females as stimulus, which were recombined into 51 pairs. Females were matched for weight (mean difference +/- SE = 0.1 +/-0.2) and standard length (mean difference +/- SE= 0.2 g +/- 0.1). Weight and standard length were combined in one measure to reflect body condition, by dividing standard length by weight. Tests began by removing the opaque PVC cylinders and lasted 30 minutes. Female species position was reversed in the second test. We tested 4 *P. pundamilia* control males, 14 *P. pundamilia* cross-fostered males, 5 *P. nyererei* control males, 11 *P. nyererei* cross-fostered males, from in total 18 broods. A focal male never encountered the same stimuli in the second test.

Ethical Note

The wild-caught stock was collected by angling and gill netting after which the fish were housed in large tanks at TAFIRI (Tanzanian Fisheries and Research Institute) in Mwanza, Tanzania. The gill netted fish were taken out of the net under water as soon as they were caught in the net by scuba divers. All the fish that were captured survived and the angle punctures in the mouth/lips and gill net marks on the dorsum healed within a few days. Fish required no medical treatment. The number of fish collected at Makobe island, 20 of each sex and each species, could not have a significant effect on population sizes, which are estimated at several thousand. Before transport to The Netherlands (by air), the fish were packed in plastic bags provided with water and pure oxygen. Fish were inspected by a Veterinarian (Fish Technologist) before transport. The Ministry of Natural Resources and Tourism in Mwanza provided an export permit, number FS/L.80/1. The fish were imported into The Netherlands under licence 250-92/257 of the National Museum of Natural History in Leiden, The Netherlands, granted by the Dutch Tax and Customs Administration. The fish arrived on 16 February 2003 in Leiden, and were moved directly to our aquarium facilities. The whole transport procedure lasted 32 hours. None of the fish died during transport or after arrival in the laboratory.

The PIT tags were implanted by inserting a hollow needle slightly smaller in diameter than the tags (which are just less than 2 mm in diameter) into the abdominal cavity and then inserting the tag into the puncture. We did not use the implantation devise recommended by the manufacturer, because those punctures are unnecessarily large. The wound was sealed with paraffin paste. The procedure took place above water and lasted up to one minute. The implantation of the PIT tags did not cause any adverse effects on the fish. We did not observe any effects on behaviour, reproduction and nuptial colouration and the small puncture wounds healed quickly without further medical treatment. We did not use anaesthetic during implantation of the tags, because in a pilot study, we found that the effect of anaesthetic lasted several days

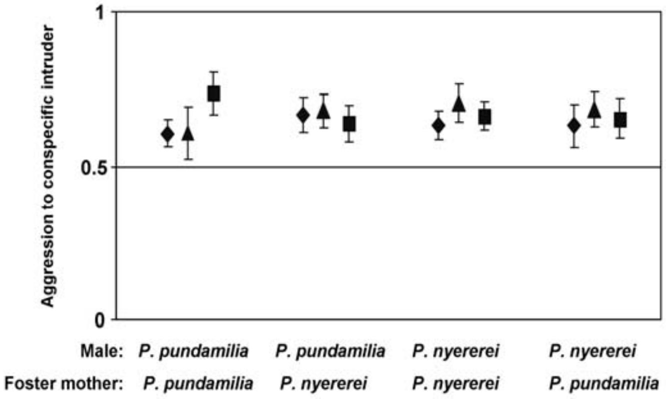

Figure 1 Male aggression test. Average proportion of aggressive behaviour directed at conspecific intruder. Diamond: time; Triangles FD-B; Squares: LD-Q. Mean +/- SEM. The horizontal line indicates equal response scores. There were no differences between the species, or treatments and all measures indicated a bias for aggression towards the conspecific intruder.

(assessed on swimming, eating and social behaviour), while unanaesthetised fish showed normal behaviour within minutes after the procedure.
During the aggression tests, males never had direct contact, and could therefore not inflict wounds on each other. Males did bump into the Plexiglass cylinder and attempted biting it as well. We did not observe any wounds or bruises on their mouths, and all males continued eating after the experiment, indicating that there were no adverse effects on their teeth and jaws. The University Committee for Animal Experiments (UDEC) approved this experiment under license number: DEC03079**.**

Statistical Analysis

All statistical analysis was done in R (R Development Core Team 2005). We fitted Generalised Linear Mixed Effect Models (GLMMs). All models were hierarchically nested, correcting for any pseudoreplication: broods within treatment, and individuals (two trials) within broods. For the analysis of the aggression biases, we had two fixed effects which each had two levels: species of the focal individual (*P.pundamilia* or *P.nyererei*) and its treatment (conspecific or heterospecific foster mother). If the fixed effect of species is significant in the model, the species respond differently in the experiments, if the fixed effect of treatment if significant, the males in the different treatments respond different in the experiments. An interaction would indicate that the treatments had a different effect in each species. In the analyses we included only one fixed factor 'species', but included both nested random effects 'brood' and 'individual'. We stepwise deleted factors from a fully saturated model until the minimal adequate GLMM was found, but always keeping repeated measures for each individual as a random factor. The factor significance levels are reported from a Chi-square test on the deviance, as appropriate for binomial data. When the minimal adequate model only included the intercept as a fixed effect (which indicates a deviation from an equal response to both stimuli), the intercept estimate is also reported.

For the aggression tests we fitted models to each of the following variables: the proportion of frontal displays and bites (FD-B) to conspecific males (relative to heterospecific males); the proportion lateral displays and quivers (LD-Q) to conspecific males; and the proportion of time spent interacting with conspecific males. For the male mate choice tests we fitted models to both the proportion of courtship events directed towards conspecific females and the proportion of the time spent with conspecific females. Because each male was tested twice in each experimental setup, we also tested for an effect of experience with that context, i.e. whether their behaviour changed between test 1 and 2.

We also tested for a relationship between aggression bias and mate choice preference of the individuals. We had data for this analysis from 15 *P. nyererei* and 18 *P. pundamilia* males; one of the *P. nyererei* males tested for mate choice was not tested for aggression bias and was therefore excluded from this analysis. For this analysis we used the average of the two tests in each setup (aggression and mate choice). Both the proportion of courtship displays to conspecific females and the proportion of time spent with conspecific females were regressed separately against the proportion of each of the two measures of aggressive displays (FD-B and LD-Q), and also against the time spent in aggressive interaction with conspecific males.

Results

Results Aggression Tests

Figure 1 shows that males of both species directed more aggression towards conspecific intruders than to heterospecific intruders. It also shows the absence of effect of the cross-fostering treatment. The full model we tested included the fixed effects: species, treatment and standard length difference. The latter was the difference in body length between the stimulus males. There was a strong preference for displaying, in both behavioural measures, towards the conspecific intruder, and also more time was spent with the conspecific intruder: fraction FD-B $F(1,39)=36.77$, $P<0.0001$; LD-Q $F(1,39)=17.68$, $P<0.0001$; time spent interacting with conspecific male: $F(1,39)=16.49$, $P<0.0001$. There was no effect of the standard length difference between the males of a stimulus pair. We found no effect of either the cross-fostering treatment or of species. See table 1 for the results and estimates of effect sizes from the GLMM.

Testing for the difference in behaviour between the two trials for each male, we found that males spent significantly more time attacking conspecific males in the second trial than in the first trial in both behavioural measures (FD-B: $F(1,78)=11.63$, $P<0.001$ with effect size 0.71 +/- 0.06 SE. LD-Q: $F(1,78)=3.3$, $P=0.05$ with effect size 0.63 +/- 0.07 SE) and time ($F(1,78=4.0$, $P=0.03$ with effect size 0.62 +/- 0.01 SE). Figure 3a shows the change between trials for the proportion time spent interacting with the conspecific intruder.

Results Male Mate Choice Tests

Figure 2 shows that it is unlikely that male mate choice was affected by the cross-fostering treatment. The main aim of this study is to address the effect of cross-fostering on male mate preferences. Due to the low sample sizes for the control groups, the statistical analysis has limited power. We therefore omitted a test for species differences in this treatment. The full model we tested included the fixed effects: treatment and body condition of the female (standard length / weight). Table 2 shows the results and estimates of effect sizes from the GLMM. We did not find an indication that the cross fostering treatment had any effect on the males' mate preferences. We found no effect of the difference in body condition within the stimuli pair in either the males' display behaviour or on the time the male spent with either member of the

	Explanatory variable	**Effect size**	**± s.e.**	**F**	**d.f.**	**p**
Full model: species x treatment + standard length difference, nested for individuals and brood						
Number of individuals: 40, from 19 broods						
FD-B	**different from equal**	0.72	0.16	36.77	1,39	**<0.0001**
	standard length difference	0.20	0.15	1.61	1,38	0.21
	treatment	0.19	0.33	0.25	1,37	0.62
	species	0.10	0.31	0.02	1,36	0.88
	species x treatment	0.19	0.77	0.06	1,35	0.80
	brood					0.31
LD-Q	**different from equal**	0.66	0.15	17.68	1,39	**<0.0001**
	standard length difference	0.13	0.16	0.61	1,38	0.44
	species	0.17	0.31	0.26	1,37	0.61
	Treatment	0.19	0.34	0.17	1,36	0.68
	species x treatment	0.23	0.70	0.10	1,35	0.75
	brood					0.28
time	**different from equal**	0.64	0.14	16.49	1,39	**<0.0001**
	standard length difference	0.06	0.13	0.20	1,38	0.66
	Species	0.06	0.28	0.04	1,37	0.85
	Treatment	0.02	0.31	0.01	1,36	0.99
	species x treatment	0.03	0.66	0.01	1,35	0.96
	Brood					0.13

Table1 Aggression analysis results of the GLMM.
Effects are listed in reverse order of removal from the model. In bold the final model, which only includes significant effects. When none of the fixed factors was significant (which was the case for each of the three variables), the analysis shows whether the variable is significantly different from equal response to both intruders. The estimates of the proportion behaviour (or time) were in all three variables significantly different from 0.5, indicating a bias towards interacting with the conspecific intruder.

stimulus pair. Interaction between female preferences for conspecific males and male preferences may result in an absence of a difference between treatments within a species. We therefore tested if females approached less to displays of heterospecific males. We found that there was no difference in the tendency to approach if the female was heterospecific or conspecific (pairwise T test t_{61}=0.338 *P*=0.736).

Since there was an absence of effect of treatment, we merged the data of the two treatment groups per species, and tested for a mate preference. There was, a hint for mate preferences in *P. nyererei* males, which displayed significantly more often to *P. nyererei* females than to *P. pundamilia* females: *F*(1,15)=9.58, *P*=0.006, estimate of proportion of displays to conspecific females 0.63 +/- 0.04 SE; and showed a trend to spending more time with *P. nyererei* females: *F*(1,15)=3.25, *P*=0.09, estimate of proportion of time spent with conspecific females: 0.55 +/- 0.03 SE. For *P. pundamilia* males, we did not find any differences in courtship behaviour or time spent with regard to the species of the stimulus females. Displays *F*(1,17)=0.13, *P*=0.72, estimate of proportion of displays to conspecific females 0.48 +/- 0.06 SE. Time spent: *F*(1,17)=1.76, *P*=0.20, estimate of proportion of time spent with conspecific females: 0.46 +/- 0.04 SE.

Testing for a change in behaviour between the first and the second trial, we found a significant interaction between trials effect and species, for both the display and time spent parameters. Display: species x trial *F*(1,64)=5.47, *P*=0.02; time: species x trial *F*(1,64)=15.68, *P*<0.001. This indicates that the males of the two species changed their behaviour differently between the two trials. To test for the main effect of trial, without an interaction effect, we tested per species separately. This showed that *P. nyererei* males increased their preference for conspecific females in the second trial compared to the first trial: displays *F*(1,30)=5.22, *P*=0.02, effect size: 0.13 +/- 0.05 SE, time spent: *F*(1,30)=13.31, *P*<0.0001, effect size: 0.07 +/- 0.02 SE. *P. pundamilia*

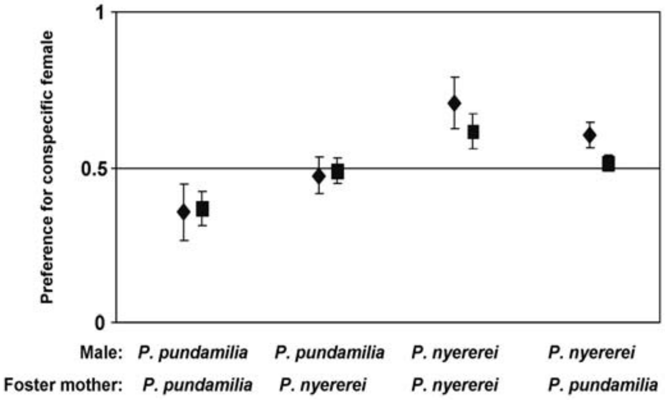

Figure 2 Male preference test. Average proportion of time and displays with conspecific female. Diamonds: displays; Squares:time. Mean +/- SEM. The horizontal line indicates equal response scores. We found no effect of treatment. There was a difference between the species: *P. nyererei* showed a preference for conspecific females, whereas *P. pundamilia* did not.

	Explanatory Variable	Effect size	± s.e.	F	d.f.	p
Full model: species x treatment + body index, nested for individuals and brood						
Number of individuals: 34, from 18 broods						
displays	Treatment	0.22	0.40	0.31	1,31	0.58
	body condition	0.05	0.20	0.06	1,30	0.81
	brood					1.00
time	Treatment	0.03	0.21	0.01	1,31	0.90
	body condition	0.02	0.12	0.12	1,30	0.89
	brood					1.00

Table2 Results of the GLMM of the mate choice experiments.
Effects are listed in reverse order of removal from the model. In bold the final model, which only includes significant effects. The two tests for each individual were always retained in the model. When none of the fixed factors was significant (which was the case for both variables), the analysis shows whether the variable is significantly different from equal response to both intruders. The estimates of the proportion behaviour (or time) were in neither variable significantly different from 0.5, indicating no species specific preference.

males showed no difference in preference measured in the displays between the two trials F(1,33)=1.37, P=0.24, effect size: 0.05 +/- 0.04 SE, but spent more time with heterospecific females in the second trial F(1,33)=2.55, P=0.01, effect size 0.05 +/- 0.02. Figure 3b (for *P. nyererei*) and 3c (for *P. pundamilia*) show the change in the proportion of time spent with the conspecific female between the two trials.

Regression of Aggression and Mate Choice Behaviour

There was no significant relation in any combination between the degree of assortative courtship displays and assortative aggressive behaviour: LD-Q: F (2,30)=0.25, P=0.761, r^2=0.02; courtship displays and FD-B: F(2,30)=0.30, P = 0.722, r^2=0.02; courtship displays and time spent interacting with intruder: F (2,30)=1.34, P = 0.232, r^2=0.08.

There was also no significant correlation in any combination of the proportion of time a male spent with a conspecific female and assortative aggressive behaviour: LD-Q: F(2,30)=0.13, P = 0.871, r^2=0.01; FD-B: F(2,30)=0.33, P=0.705, r^2=0.02, time spent with conspecific female and time spent interacting with conspecific intruder: F(2,30)=0.16, P=0.844, r^2=0.01.

Discussion

With a cross-fostering experiment, we tested whether imprinting on the maternal phenotype mediates male mate preferences and aggression biases in two closely related species of Lake Victoria cichlids. Imprinting may yield

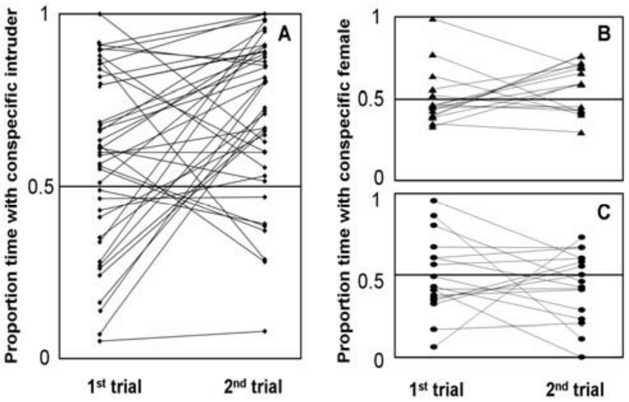

Figure 3 Differences between trials. The lines connect the two scores of an individual. **A**. Aggression tests of both species. Proportion of the time spent interacting with the conspecific intruder increased from trial 1 to trial 2. **B** *P. nyererei* mate choice trials. Proportion of the time spent with the conspecific female increased with trial 1 to trial 2. **C**.*P. pundamilia* mate choice trials. Proportion of the time spent with the conspecific female decreased slightly from trial 1 to trial 2.

strong assortative behaviour, which is critical for species coexistence and also for sympatric speciation. There was no evidence that males imprinted on their mothers' phenotype for their later aggression bias. Male aggression in territorial defence was directed primarily towards conspecific intruders, in males of both species and of both treatments. They showed therefore the same aggression bias as males showed in the wild, and wild caught males in laboratory experiments (Dijkstra et al. 2006b). However, we did find that the males' behaviour changed with experience, since they showed a stronger aggression bias to conspecific males in the second trial.

It is also highly unlikely that imprinting affects male mate preferences for conspecific females. An interaction with the females' preferences could obscure male mate preferences, if males stop displaying to females that are uninterested. However, there was no difference in response ratio between the two females in a mate choice test, and the response ratio of the females was quite high. Although there may be other, more subtle cues from the female that we were not able to measure, from our data it seems unlikely that males may have displayed against their preference due to the females' behaviour. We found some indication that *P. nyererei* males have a preference for conspecific females. Pierotti & Seehausen (2007) showed evidence for morph specific male mate preferences in a Lake Victoria cichlid species (*Neochromis omnicearuleus*) which shows three distinctly different morphs. It is therefore not unlikely that males of *P. nyererei* show preferences for females of their own species over heterospecific, congenic females. However, we point out that our sample sizes are quite low. While the overall effect of a lack of an effect of cross-fostering on mate choice seems quite robust, the data do not allow a more fine tuned analyses of biases or species differences, and any firm conclusions on these issues are at the moment premature. Finally, we found no relation between a bias in a male's territorial defence and his mate choice behaviour. We tested for this because a males' preference for conspecific females may have implications for its perception of territorial competitors, or vice versa, however our results provide no evidence for this. It can be difficult to test for such relationships, since this requires that individuals' experiences are controlled until the time of testing. Although the aim of this study was not to test for such a behavioural coupling, since the males had only had experience with other males until their first mate choice test, this study provided an opportunity.

The absence of an effect of cross-fostering in both male mate choice and male territorial defence contexts is in contrast with the finding that females do show sexual imprinting on their mother (chapter 3). Our conclusion is that the sexes show a difference in development for species recognition in sexual contexts. Such results bear some resemblance to those of Barlow (1992), who found that males of the Middle American Midas cichlid (*Cichlasoma citrinellum*) also show no signs of imprinting, however he found no strong effects of sexual imprinting in females. Sex differences in sexual imprinting have also been reported for several bird species (e.g ten Cate 1985; ten Cate & Vos 1999; Witte & Sawka 2003). Many of these may be explained by the use of different cues for partner selection by the two sexes (ten Cate 1985; ten Cate & Vos 1999). Preferences for such different cues (such as body size, colour pattern, colour intensity, behaviour, sounds etc.) may be affected in different ways by early development (ten Cate 1985). The apparent difference between the sexes in the cichlid species in this study may have a similar underlying explanation, because females and males of these species are sexually dimorph. For instance, females exhibit a much lower level of coloration compared to the males, and males and females also differ in other morphological aspects (Seehausen et al. 1998; chapter 3). Thus, males and females may, due to the different cues available to both sexes, use different cues for mate selection.

Although an absence of evidence for imprinting in the males in this study may not be evidence of absence of imprinting, a difference between the males and females for the effect of cross fostering may make sense in the light of skewed reproductive potential. The asymmetry in reproductive investment in these lekking species would predict that females are under strong selection to be choosy and males to be less discriminating (Trivers 1972). Female cichlids may therefore have endured stronger selection pressures to develop a mechanism for assortative mating. However, there is accumulating evidence for male mate preferences in lekking species (Saether et al. 2001; Werner & Lotem 2003; Werner & Lotem 2006; Pierotti & Seehausen 2007). For instance in lekking great snipe *(Gallinago media)*, males may forgo matings with a female he already mated with, in favour of a future opportunity (Saether et al. 2007). Such male mate preferences in lekking species indicate that males also have a limited reproductive potential, and therefore can be expected to show some choosiness. Though interspecific mating between *P. nyererei* and *P. pundamilia* produces viable and fertile offspring (Seehausen et al. 1997, van der Sluijs *in* *press*), such hybrid offspring may be less attractive and hence suffer from a decreased fitness. *Pundamilia nyererei* males may therefore forgo such mating opportunities in favour of species assortative matings.

If male territorial aggression biases and male mate preferences are not mediated by sexual imprinting, the question remains open as to what mechanism does mediate them. Male territorial aggression biases in these species can be influenced by experience, which has been shown in a study on the same species (Dijkstra et al. 2006a). Interestingly, we found an increase in assortative male-male aggression from the first trial to the second, also suggesting an influence of experience on male aggression (figure 3a). Also for the mating preferences we observed an effect of experience, at least in *P. nyererei*, which showed an increase in assortative mate preferences for *P. nyererei* females from the first trial to the second (figure 3b). In another study, males that had experience with males from both species showed more aggression towards their own species, while males only exposed to their own species showed no such bias (Dijkstra et al. 2006a). Such results suggest that experience with other species may be necessary for the development of an aggression bias. The results of our study, however, show that this need not be the case, because the males in our experiment, which were raised by a conspecific female, had never been in contact with males from the other species, yet they showed more aggression towards conspecifcs. Another type of experience that could have affected male behaviour is that males in this experiment may have learnt about their brothers' phenotype. The full broods were cross-fostered, and males were kept with their male siblings until the time of testing. In another study on a species pair of a different genus we show that such an effect can occur (chapter 5). Here we show that males do not show a difference in aggression bias as a result of experience with a foster mother of a different species. Alternatively, a predominantly genetic background for differences in territorial defence biases and mate preferences may also explain the observed behaviour, not withstanding the indication that adult experience may shape territorial defence behaviour in these cichlids. More experiments are needed in order to rule out these alternative hypotheses.

Both species showed clear species assortative behaviour in male-male interactions. The strong assortative character of male-male aggression may contribute to frequency dependent intra sexual selection, which is suggested to have contributed to the sympatric occurrence of haplochromine cichlids in general (Mikami et al. 2004; Seehausen & Schluter 2004) and for *P. pundamilia* and *P. nyererei* in particular (Dijkstra et al. 2005; 2006b; 2007). If male aggression biases are at least partly formed by adult experience, which seems to be the case (this study and Dijkstra et al. 2006a), then they can adjust, from one generation to the next, to a changing composition of species and phenotypes in the population. Such flexible aggression biases may be counter to a frequency dependent intra sexual selection scenario.

Strong male mate preferences for conspecific females would aid the development of assortative mating (Almeida & de Abreu 2003; van Doorn et al. 2004), and would thus make a scenario of sympatric speciation more likely. The species specific male mate preferences in *P. nyererei* may help this way. However, our findings give some indication that their preferences may be affected by courtship experience, in which case the male mate preferences could be indirectly shaped by those of the females. While the net result is species assortative mate preferences in both sexes, a developmental mechanism independent of encounters with unrelated females would probably promote reproductive isolation more straightforwardly. To conclude, although these results contribute to the understanding of how the species assortative behaviour may have arisen in these cichlids, the picture is still far from complete.

Acknowledgements

Comments by Cerise Allen, Peter Dijkstra, Marie-Jeanne Holveck and Inke van der Sluijs, Michael Ryan and two anonymous reviewers greatly improved this manuscript. We thank Peter Dijkstra, Enja Feuth de Bruin, Mohammed Haluna, Kees Hofker and Mhoja Kayeba for help at various stages of the experiment. This study was financially supported by NWO (ALW-810.64.012) and Lucie Burgers Foundation for Comparative Behaviour Research, Arnhem, The Netherlands.