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Reproductive isolating barriers between colour-differentiated populations of an African annual killifish, *Nothobranchius korthausae* (Cyprinodontiformes)

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Allopatric populations separated by vicariance events are expected to evolve reproductive isolating mechanisms as a result of disparate selection pressures and genetic drift. The appearance of reproductive isolating mechanisms may vary across taxa with differences in the opportunity for mate choice, and may be asymmetrical. In addition, premating barriers may be affected by individual mating experience. We used choice and no-choice experiments to investigate reproductive isolation between two allopatric (island and mainland) and colour-differentiated populations of an African annual fish, *Nothobranchius korthausae*. Assortative mating under experimental conditions was limited and asymmetrical. Preference for sympatric males was only expressed in nonvirgin females from one population. Virgin fish from both populations mated indiscriminately. No difference in the number of eggs laid, fertilization rate and hatching success was detected in no-choice experiments. All mating combinations produced viable offspring and no postmating barriers were detected in terms of the performance and fertility of F1 hybrids. Overall, we found little evidence for significant reproductive isolation, which is in contrast with the related killifish taxa in which assortative mating can be strong, even among allopatric populations with no colour differentiation. *Biological Journal of the Linnean Society*, 2010, **100**, 62–72.

ADDITIONAL KEYWORDS: allopatric divergence – assortative mating – colour polymorphism – mating experience – premating barriers – reproductive isolation.

INTRODUCTION

The understanding of how new species arise and how they remain separate from other species is a major goal of current research in evolutionary biology. Regardless of the exact species' definition within disparate species' concepts, species are considered to constitute pools of interbreeding individuals that are substantially reproductively isolated from other such pools (Coyne & Orr, 2004; Mallet, 2008). Reproductive isolation is manifested through reproductive isolating barriers that decrease the probability of offspring production between individuals from different species' pools.

Allopatric populations that have been isolated by vicariance events are predicted to diverge as a result of disparate selection pressures and genetic drift. Many closely related species with current sympatric occurrence have supposedly evolved in allopatry, where they diverged and reproductive isolating barriers evolved, resulting in reproductively isolated entities after cessation of the physical barrier between their ranges (Hewitt, 2000). The diversification rate of clades diverging in allopatry may be as rapid as diversification in sympatry (Near & Benard, 2004).

Sexual selection can increase the speed of reproductive isolation via the generation of assortative mating (Nosil & Yukilevich, 2008) based on particular visual, auditory or olfactory cues. Post-zygotic barriers tend to accumulate when populations are isolated,

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*Corresponding author. E-mail: reichard@ivb.cz and secondary contact then generates selection for assortative mating. Although sexual selection is more often studied as an important source of divergent selection in speciation processes occurring in sympatry or parapatry, where assortative mating is required to evolve at very early stages of divergence (Seehausen, 2006), it plays a similarly important role in allopatric speciation. For example, *Tropheus* cichlids from Lake Tanganyika are likely to have diverged in multiple allopatric populations during periods of lower water level in the lake (Sturmbauer & Meyer, 1992) and most populations have evolved different coloration. At present, there is no natural secondary contact between the colour morphs, but analysis of accidentally admixed colour-differentiated populations showed colour-assortative mating in nature (Salzburger *et al.*, 2006), and laboratory experiments have demonstrated full reproductive isolation among populations (Egger *et al.*, 2008).

Reproductive isolation can be asymmetrical (Smadja & Ganem, 2005) when one population (or species) mates assortatively, but the second population/species mates either indiscriminately (Strecker & Kodric-Brown, 1999) or even prefers partners from allopatric populations/species (Ryan & Wagner, 1987; Rosenfield & Kodric-Brown, 2003; Wong, Fisher & Rosenthal, 2005), which is often the case if sexual selection drives mating patterns (Basolo, 1990; Kodric-Brown & Strecker, 2001). Apart from direct signals, mate choice may be based on a partner's dominance status (Andersson, 1994). The strength of mate choice may also depend on the reproductive experience of a particular individual. Virgin females mate less discriminately than experienced females (theory: Kokko & Mappes, 2005; wolf spiders: Hebets & Vink, 2007), with virgin choosiness decreasing, in particular, after prolonged inability to lay already ovulated eggs (cockroaches: Moore & Moore, 2001).

Here we use a small species of African annual fish, *Nothobranchius korthausae* Meinken 1973, to test the hypotheses of assortative mating and incipient speciation between two populations. The two populations were geographically separated by a vicariant event during an increase in sea level in the Indian Ocean at least 7000 years ago (Camoin *et al.*, 1997; Fleming *et al.*, 1998). The timing of this event is comparable with recent water level changes in the major African lakes (Johnson *et al.*, 1996) and other rapidly radiating fish study systems, such as killifish genera *Cyprinodon* in Laguna Chichancanab (Strecker, 2006) and *Cynolebias* occurring on the coastal plains of Uruguay (García, 2006). Different taxa have different speciation dynamics (Hendry, 2009) and *Nothobranchius* may therefore provide a useful system in studies on assortative mating and incipient speciation.

Nothobranchius korthausae is a small (total length, 5 cm), sexually dichromatic fish inhabiting isolated pools in a swampy coastal region of mainland Tanzania (the lower Rufiji river basin including adjacent minor coastal river systems) and on Mafia Island, just off the Rufiji delta. Two colour morphs occur in male *N. korthausae*: the yellow morph is found throughout the species' range, and the red morph is constrained to the northern and central part of Mafia Island (Wildekamp, 2004). The colour morphs are typically allopatric, although sympatric occurrence has been reported from several sites in the northern and central parts of Mafia Island (Wildekamp, 2004). The existence of two colour morphs is common in most *Nothobranchius* species, but nothing is currently known on how they have evolved and coexist (Wildekamp, 2004; Reichard, Polačik & Sedláček, 2009). Based on individuals from some (northernmost) mainland populations, a separate species, called *Nothobranchius ruudwildekampi* Costa 2009, has been described recently on the basis of different coloration (Costa, 2009). The Kwachepa population of *N. korthausae* used in the present study shares most diagnostic characters with *N. ruudwildekampi*, but we tentatively keep the two populations conspecific pending further (e.g. molecular) evidence of the validity of *N. ruudwildekampi*. So far, it appears that *N. ruudwildekampi* has been described on the basis of one extreme of the continuous cline in coloration (M. Reichard, pers. observ.).

Nothobranchius korthausae constitutes a useful model system for studies related to reproductive behaviour; like all *Nothobranchius* species, *N. korthausae* spawns daily after reaching sexual maturity, its mating system is promiscuous and no pair bonds are formed (Haas, 1976a; Wildekamp, 2004). Males compete with each other for access to females, but no territories are formed (Haas, 1976b). Males possess a bright nuptial coloration that is the target of female choice, at least in a closely related *N. guentheri* (Haas, 1976b). Female coloration is drab (uniformly brown). Females lay 5–50 eggs each day in separate clutches of single eggs (Polačik & Reichard, 2009). The life cycle is annual; adults die during the dry season and populations persist in the form of eggs deposited in the substrate.

We investigated whether the two populations of *N. korthausae* have begun to evolve reproductive isolating barriers. First, we tested whether assortative mating has evolved and how assortative mating is affected by previous mating experience. Secondly, we investigated post-mating barriers in the form of fertilization rate and hatching success. Finally, we tested the performance (fecundity, hatching success) of the F1 generation from allopatric (interpopulation) and sympatric crosses. We hypothesized that premating reproductive isolating barriers would be manifested in the form of partial assortative mating, virgin fish would mate less discriminately than nonvirgin fish, and post-mating barriers would be incomplete, but with some decrease in hybrid performance.

MATERIAL AND METHODS

Experimental animals

Experimental fish originated from two populations of *N. korthausae* that were collected in 2001 (mainland population from Kwachepa, yellow males) and 2002 (population from Mafia Island, red males) (for details on populations, see Polačik & Reichard, 2009).

Experimental fish were raised under two regimes (virgin and nonvirgin fish). The eggs that were stored in a mixture of peat moss and coconut fibre were rinsed with dechlorinated tap water. Fish that hatched within 6 h were transferred into 40-L tanks filled with 10 L of water with an addition of 3 L of water daily up to 40 L, and fed with *Artemia* nauplii. To obtain a set of virgin fish, any juvenile with a sign of male coloration (that starts to develop 2 weeks prior to sexual maturity) was separated into male tanks. Fish coloration was checked at least three times a week. All tanks set to raise virgin experimental fish were separated by opaque barriers to prevent any fish interaction with sexually mature fish of the opposite sex prior to experimental trials. Subadult and adult fish were raised in same-sex groups of 10–15 fish in 40-L glass aquaria with a 14-h : 10-h light : dark cycle at a temperature of 25 °C, and fed with a mixture of live and frozen *Chironomus* and *Chaoborus* larvae. The same procedure was followed in rearing nonvirgin fish, except that the fish were kept in mixed-sex groups and interacted freely (including spawning) before experiments.

Experimental procedures

Experiments were conducted separately for virgin and nonvirgin fish. Testing followed the same protocol, except for housing conditions. Thirty-two virgin and 28 nonvirgin females and males were used. First, we performed mate choice experiments and scored female and male behaviours, with a single female choosing between two males (sympatric, allopatric). The same two males were then tested with another female (from the second population). This same set of four fish formed an experimental group for no-choice experiments, where each female was allowed to spawn sequentially with two males (sympatric, allopatric). From the no-choice experiment, eggs were collected, counted and their fertilization rate and hatching success were scored. We further associated the behavioural preference of females in choice tests with their preference based on the number of eggs produced in no-choice tests. We then raised the offspring to adulthood and tested their performance (egg production and hatching rate).

Mate choice experiments consisted of dichotomous tests in which a female was able to choose between two males. Tests were undertaken in 40 cm × 25 cm × 20 cm (depth) tanks consisting of three compartments of equal size divided by transparent plastic partitions. The outer (male) compartments were equipped with Petri dishes (diameter, 10 cm) containing a thin layer of fine sand (spawning substrate) that served as the centre of their territories. Partitions were perforated and visual, olfactory and acoustic communication among fish within the tank was possible, although each experimental tank was separated by a 3-cm-thick Styrofoam barrier to prevent any kind of communication between fish from different tanks. A total of 30 min of fish behaviour was scored for each replicate. Males (one from each population) were moved into the outer sections in a random, predetermined position (left or right) and a female was placed in the middle section after 30 min and allowed to settle. Then, fish behaviour was scored in three 10-min intervals during the following hour. For each 30 s of the recording period, the following behaviours were scored as present or not: male courtship (male approaches female with a characteristic darting movement, followed by a brief series of contacting movements with his head slightly upward and exposing his lateral side) and female response (slow female approach to the particular male, followed by an attempted interference with the male). The described behaviour is unambiguous and forms the typical prelude to spawning in *Nothobranchius* (Haas, 1976a).

The same procedure was used to record the behaviour of the second female. When recording was complete, the female was placed in a separate tank. A new Petri dish with a spawning substrate was then placed into the central compartment. Male partitions and Petri dishes were removed and dominance between males was scored in the absence of females. *Nothobranchius* males are known to compete aggressively with each other and are in constant readiness to spawn (Haas, 1976a, b). One of the males quickly established dominance, evident by chasing the other male away from the spawning substrate and pressing the subordinate male downwards after clasping him with the dorsal fin. In three cases, dominance was not established as the contest between similarly strong males had to be terminated following the approved experimental protocol to avoid any injury.

No-choice experiments followed immediately after the choice test and dominance trials. Each tested male was housed with one tested female in a 40-L tank with a spawning substrate (fine sand) in a Petri dish and left to spawn for 20 h. After 20 h, the Petri dish was carefully removed and the eggs were washed out and counted. Males and females were separated for 24 h and then placed with the second partner from the experimental group and allowed to spawn for 20 h. A period of 24 h is sufficient for females to fully recover egg mass (Polačik & Reichard, 2009; M. Polačik and M. Reichard, unpublished pilot studies). At the end of the trial, each fish was measured from the tip of the snout to the margin of the caudal fin to the nearest 0.5 mm in a glass cell with a ruler.

Fertilization success was estimated by counting the number of developing eggs after 48 h. During egg counting, every egg was placed in a separate cell of Nunc Multidish culture plasticware (cell diameter, 20 mm) filled with tank water to a depth of 5–10 mm. After 48 h, every egg was individually observed under a dissecting microscope and scored as developing (transparent, chorion separated from yolk – perivitelline space obvious) or dead (opaque, often with fungal infections). We cannot exclude the possibility that some eggs scored as 'fertilization failed' were actually fertilized, but died during the initial stages of their development.

Hatching success was estimated as the proportion of eggs scored as 'fertilized' that successfully hatched. After scoring fertilization success, the eggs were placed into a coconut fibre substrate and stored in plastic bags in the dark at room temperature (23–26 °C). Eggs from each replicate were stored in a separate bag. After 6 weeks, the substrate was wetted with dechlorinated tap water and all hatched fish were counted after 12 h. The substrate was then dried and watered for a second time after an additional 2 weeks, as not all *Nothobranchius* eggs develop at the same rate (Wourms, 1972).

Hybrid performance experiments were carried out on experimental offspring and offspring from additional breeding involving nonexperimental fish. Juveniles from multiple families (maximum of eight juveniles per family) were pooled after hatching as a result of logistic constraints. Fish of all four treatments were reared (male Mafia × female Mafia; male Kwachepa × female Kwachepa; male Mafia × female Kwachepa; male Kwachepa × female Mafia) following the protocol for nonvirgin fish (i.e. no sex separation). As we were primarily interested in postzygotic measures of hybrid performance in the F1 generation, only no-choice trials were performed. A modified procedure was applied as groups of four males and four females were tested. The experiment consisted of successive pairings of each experimental male with each experimental female within the group. First, the female spawning condition was standardized by allowing each female to spawn with a nonexperimental male. After 45 h, each female was allowed to spawn with one male for 3 h, followed by a 45-h resting period before her second spawning with another male. During subsequent experimental trials, experimental females were assigned to a different experimental male from the group each time, following a random, predetermined order. A longer interspawning interval was used in this experiment because pilot studies showed that females needed a longer egg mass recovery time after spawning with more than two males. The number of eggs produced and the hatching success were estimated as described above, but fertilization success was not estimated. A total of eight groups was tested, representing 32 males and 32 females, each mated with four partners, resulting in 128 mating combinations.

STATISTICAL ANALYSIS

Differences in male courtship vigour and female response to a particular male were tested using general linear models (GLMs), with three factors (sympatry/allopatry, dominant/subordinate and male body size as continuous factor). Virgin and nonvirgin fish were tested separately. Female response was square root transformed to meet the assumption of a normal distribution. For analyses of the number of eggs laid (quasi-Poisson distribution), fertilization rate (arcsine-transformed data) and hatching success in the no-choice test, GLMs with three factors (virgin/nonvirgin; sympatry/allopatry; dominant/subordinate) were used. We further tested for congruence between assortative association and subsequent egg production of a particular pair using a chi-squared test. A two-factor GLM (population identity; spawning rank) was used to test the performance of F1 fish (number of eggs, hatching success). The measure of hatching success was independent of egg production as it was calculated as a proportion of the fertilized eggs (or total eggs in F1 fish) that hatched successfully. Replicates that produced less than five eggs were not included in the hatching rate analysis. Mean ± SE values for the quasi-Poisson distribution were back-calculated from the exponential values used in the analysis (log link function). Statistical analyses were performed using Statistica for Windows 8.0, except for GLM with a quasi-Poisson distribution which was analysed using R package 2.9.1 (R Development Core Team, 2006).

RESULTS

CHOICE EXPERIMENTS

The effects of sympatry, male dominance and male body size on male courtship rate and female response to courtship are summarized in Table 1. In virgin fish,

Table 1. The results of general linear model (GLM) analyses of the effects of sympatry, male dominance and male body size on male courtship rate and female response to courtship

	Courtship				Response			
Female identity	*F*	d.f.	*P*		*F*	d.f.	*P*	
Virgin fish								
Kwachepa females								
Sympatry	1.96	1,24	0.174		0.03	1,24	0.875	
Dominance	0.66	1,24	0.423		0.02	1,24	0.896	
Body size	1.14	1,24	0.296		0.35	1,24	0.562	
Mafia females								
Sympatry	0.00	1,24	0.974		3.83	1,24	0.062	
Dominance	0.02	1,24	0.900		0.04	1,24	0.848	
Body size	0.36	1,24	0.554		0.18	1,24	0.673	
Nonvirgin fish								
Kwachepa females								
Sympatry	5.45	1,24	**0.028**	A > S	0.77	1,24	0.388	
Dominance	0.06	1,24	0.807		0.35	1,24	0.561	
Body size	0.92	1,24	0.346		1.17	1,24	0.291	
Mafia females								
Sympatry	4.99	1,24	**0.035**	S > A	24.98	1,24	**< 0.001**	S > A
Dominance	0.70	1,24	0.411		2.37	1,24	0.137	
Body size	1.05	1,24	0.315		1.36	1,24	0.255	

The statistical tests concern female identity, i.e. courtship received from males and female response. Male response was square root transformed to achieve a normal distribution. Significant differences are indicated in bold and the direction of preference is shown in a separate column [A > S, stronger courtship received from allopatric males; S > A, stronger courtship from (or stronger response to) sympatric males].

sympatry had no significant effect on male courtship rate or female response. However, nonvirgin Mafia males courted females from both populations significantly more vigorously and the response of Mafia females to sympatric male courtship was almost three times as strong as to allopatric male courtship. In Kwachepa females, there was no difference in response to male courtship between sympatric and allopatric males (Table 1). This result was probably mediated by the very strong responsiveness of nonvirgin Mafia females to sympatric males rather than the generally low responsiveness to male courtship in nonvirgin Kwachepa females. The general responsiveness of nonvirgin Mafia females was twice as high as the responsiveness of nonvirgin Kwachepa females (*t*-test, $t_{26} = 5.15$, $P < 0.001$), but there was no difference in general responsiveness between virgin females from Kwachepa and Mafia (*t*-test, $t_{28} = 0.36$, $P = 0.723$). General responsiveness in nonvirgin Mafia females was twice as high as in nonvirgin Kwachepa females and virgin Mafia and Kwachepa females (Fig. 1).

Males from one population were not consistently dominant over males from the other population ($\chi^2 = 1.14$, d.f. = 1, $P = 0.285$ for nonvirgin males and $\chi^2 = 2.57$, d.f. = 1, $P = 0.109$ for virgin males). There was no association between body size and dominance (paired *t*-tests: $t_{13} = 0.32$, $P = 0.758$ for virgin males; $t_{12} = 0.56$, $P = 0.585$ for virgin males), although the body size difference was relatively minor (median, 2 mm; range, 0–8 mm). There was no association between male dominance or body size and courtship rate and female response in either virgin or nonvirgin fish (Table 1).

NO-CHOICE EXPERIMENTS

Fish spawned in 76 of 124 experimental trials (61.3%), and there was no difference between virgin (61.8%) and nonvirgin (60.7%) females, or between females from Kwachepa (61.5%) and Mafia (64.5%) populations. The overall mean number of eggs laid during the no-choice experiment was 12.6 ± 1.1 ($N = 124$). There was no effect of sympatry (GLM with a quasi-Poisson distribution, $F_{1,121} = 0.1$, $P = 0.878$) or of any interaction involving sympatry (all $P > 0.40$) on the number of eggs laid during experimental crosses (Fig. 2, left).

The mean fertilization rate was 70.8 ± 3.7% ($N = 63$). There was no significant effect of sympatry (GLM, data arcsine transformed, $F_{1,55} = 0.3$, $P = 0.599$; Fig. 2, middle) or any other factor or interaction (all

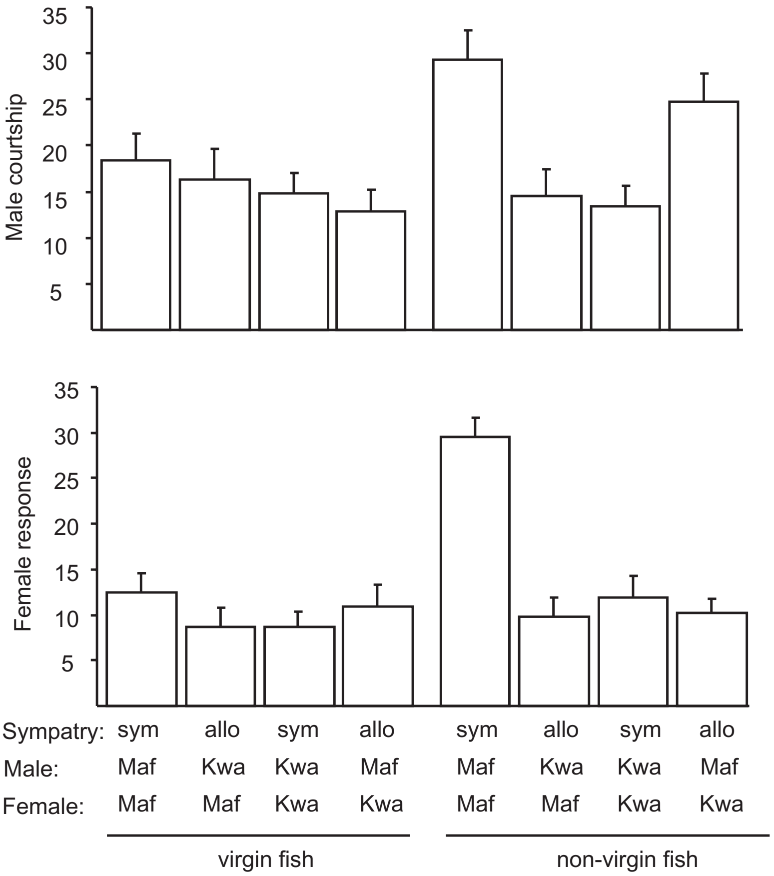

Figure 1. The rates of male courtship towards females and female response (per 30 min) expressed for each male × female combination and virgin and nonvirgin fish separately. Means with 1SE are indicated.

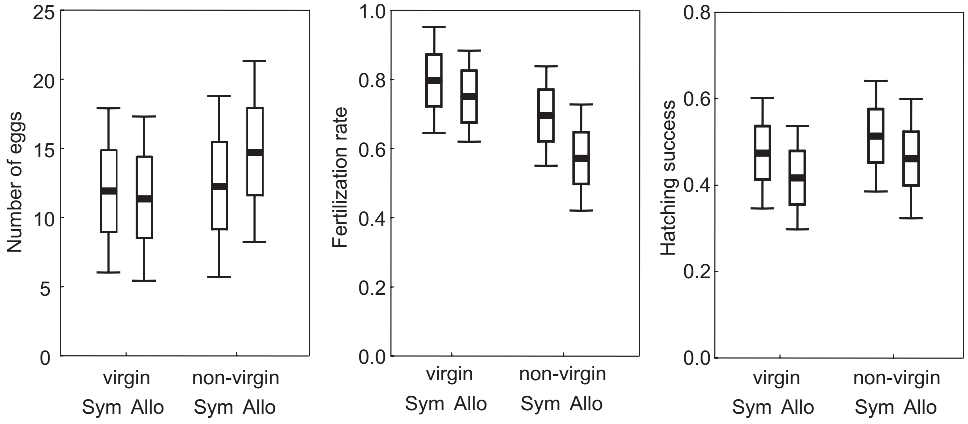

Figure 2. The number of eggs, fertilization rate and hatching success of pairings between sympatric and allopatric populations during the no-choice experiment. Means with SE (boxes) and confidence intervals (whiskers) for data on virgin and nonvirgin fish are indicated.

$P > 0.18$) on the fertilization rate. Overall, the mean hatching success was 46.5 ± 3.1% ($N = 56$). The hatching rate was not affected by sympatry ($F_{1,48} = 2.5$, $P = 0.121$; Fig. 2, right) or any interaction involving sympatry (all $P > 0.08$).

There was no match in female preference between choice and no-choice experiments in virgin fish ($\chi^2 = 4.8$, d.f. = 1, $P = 0.201$). However, a significant relationship was found in nonvirgin fish ($\chi^2 = 1.6$, d.f. = 1, $P = 0.028$), indicating that nonvirgin females associated more often with males that consequently received more of their eggs in no-choice trials.

HYBRID PERFORMANCE

The overall mean number of eggs laid during the F1 performance experiment was 22.2 ± 1.2 ($N = 128$). There was no effect of population identity of female (GLM, $F_{3,91} = 2.1$, $P = 0.107$) or male (GLM, $F_{3,91} = 1.4$, $P = 0.255$) on the number of eggs laid, nor was there any interaction between male and female population identity (GLM, $F_{9,91} = 1.8$, $P = 0.089$; Fig. 3A).

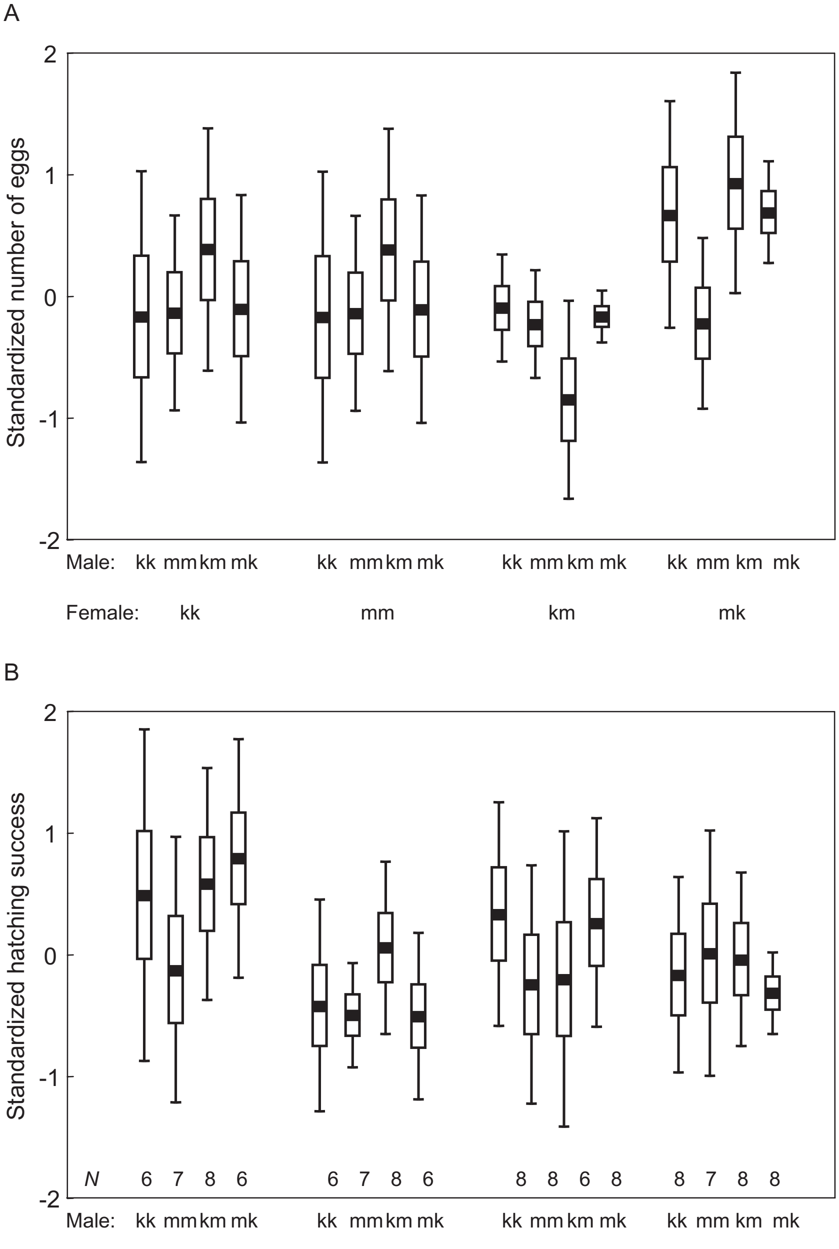

Figure 3. The results of the hybrid performance experiment, with number of eggs (A) and hatching success (B) standardized within each experimental group shown. Means with 1SE (boxes) and confidence intervals (whiskers) are indicated for each experimental combination (mm, offspring of Mafia male and Mafia female; kk, offspring of Kwachepa male and Kwachepa female; mk: Mafia male, Kwachepa female; km: Kwachepa male, Mafia female). Sample size for the number of eggs is $N = 8$ replicates for each combination, sample size for hatching success is smaller (because only replicates with more than 5 eggs were analysed) and the exact N is given above each combination.

The mean hatching success was 18.4 ± 1.7% ($N = 113$, replicates with less than five eggs were excluded). There was no effect of population identity for female (GLM, $F_{3,91} = 2.4$, $P = 0.071$) or male (GLM, $F_{3,91} = 1.4$, $P = 0.250$) on the number of eggs laid, nor was there any interaction between male and female strain identity (GLM, $F_{9,91} = 0.7$, $P = 0.732$; Fig. 3B).

DISCUSSION

We investigated reproductive isolation between two allopatric and colour-differentiated populations of an annual fish using experiments on: (1) premating barriers caused by male and female mating behaviour in mate choice tests, and (2) postmating barriers inferred through fertilization rate, hatching success and hybrid performance during no-choice tests. Overall, we detected no assortative mating preferences in virgin fish. In nonvirgin fish, we found a strong preference by females from the Mafia population to mate with sympatric males, but no consistent preference in Kwachepa females.

Premating barriers are typically expressed as an investment in courtship effort in males (Werner & Lotem, 2003) and selective mate choice in females (Endler & Houde, 1995). In our mate choice experiments, males courted females vigorously and females readily responded to male courtship in both virgin and nonvirgin fish. Nonvirgin males from the Mafia population courted all females more vigorously, and this was associated with a stronger response from sympatric (but not allopatric) females (Table 1). Stronger courtship effort often results in a stronger female response, because the two behaviours are often correlated, with female response eliciting more male courtship (Takahashi *et al.*, 2008). In contrast, no assortative mating was detected in virgin fish. This is congruent with the fact that virgin individuals are generally less discriminating (theory: Kokko & Mappes, 2005; cockroaches: Moore & Moore, 2001; wolf spiders: Hebets & Vink, 2007), and we suggest that care should be taken when virgin individuals are used in studies on assortative mating.

Association time is a commonly used measure of mate preference and has been proven to be valid in many taxa (reviewed in Goncalves & Oliveira, 2003), but not in others (Gabor, 1999; Egger *et al.*, 2008). Our data demonstrated that it appears to be a suitable measure in *Nothobranchius* fishes. In nonvirgin fish, we observed a significant correspondence between the outcomes of choice and no-choice experiments: females laid more eggs with males that were preferred in the preceding choice trial. In contrast, no such correspondence between the two experiments was observed in virgin fish. This further supports our assumption that virgin fish mate indiscriminately. An alternative explanation of the observed mating pattern is that female preference has a learned component, and females learned preference for the phenotype of males housed with them prior to the experiments. Although our experimental approach cannot differentiate unambiguously between the effects of mating experience and sexual imprinting (which would require a cross-fostering approach; Verzijden & ten Cate, 2007), the lack of female preference in nonvirgin Kwachepa females does not support this alternative explanation.

The strength of mate preference was asymmetrical, with nonvirgin females from the Mafia population choosier than females from the Kwachepa population (Table 1). Given that two colour morphs occur on Mafia Island, but not on the mainland (source of the Kwachepa population), it could be speculated that coloration divergence may have started on Mafia Island, and hence Mafia females are more discriminating. More populations for behavioural tests and genetic data are needed to test this and alternative scenarios. We are also aware that our study design allowed the simultaneous operation of visual, olfactory and auditory signals on assortative mating and did not aim to differentiate their relative importance.

No-choice tests did not result in an assortative pattern of egg allocation with regard to sympatry/allopatry. No-choice situations are frequently used to test reproductive isolation in fish and other model systems (e.g. Boughman, Rundle & Schluter, 2005; Kullmann & Klemme, 2007), although their efficacy seems to vary among particular species and mating systems. Coercive mating, a characteristic of *Nothobranchius* males, may artificially increase the level of hybridization in the laboratory, especially in the absence of a sympatric/conspecific partner (Turner *et al.*, 2001). There are examples of sympatric species being completely reproductively isolated in nature, but producing viable hybrids in the laboratory (reviewed in Knight *et al.*, 1998). Another plausible explanation of the lack of assortative egg allocation may relate to the *Nothobranchius* mating system. *Nothobranchius* females spawn several times each day, and a lack of mating partners may decrease the female acceptance threshold in order to lay the eggs she has already ovulated. Finally, in spite of the expression of female preference, opportunities for female choice may be reduced in mating systems dominated by male coercion (Reichard *et al.*, 2005), as appears to be the case in *Nothobranchius* (Haas, 1976a), and might be further intensified as a result of space limitation under experimental, but also natural, (Reichard *et al.*, 2009) conditions.

We detected no decrease in F1 hybrid performance. Hybrid performance is expected to decrease with emerging reproductive isolation, arising from gametic or genotypic incompatibilities accumulated through genetic drift (Coyne & Orr, 2004). Although a decreased fertility of F1 hybrids between closely related taxa has been observed as a result of incipient speciation (e.g. house mouse: Macholán *et al.*, 2007), many hybridizations between populations/species more divergent than our study populations have not resulted in measurable intrinsic postzygotic isolation in F1 and F2 hybrids; in those cases, a fitness decrease in hybrids may only appear several generations beyond F1 (Turner *et al.*, 2001; Price & Bouvier, 2002). In addition, negative heterosis may perhaps only be expressed in challenging natural conditions and under particular environmental settings, and therefore is less likely to be detected under benign laboratory conditions (Coyne & Orr, 2004).

We conclude that the observed reproductive isolating barriers in *N. korthausae* are relatively weak and only manifested as asymmetrical assortative mating. This is in contrast with female *Chromaphyosemion*, another species-rich, closely related genus of killifish, in which a strong and symmetrical preference for sympatric males was found in spite of almost identical colour patterns in the two studied populations (Kullmann & Klemme, 2007). It is notable that colour polymorphism in a closely related *Nothobranchius furzeri* is controlled by a simple mechanism (Reichard *et al.*, 2009; Valenzano *et al.*, 2009), similar to African lake and riverine cichlids (Koblmüller *et al.*, 2008; Seehausen *et al.*, 2008). The genetic differences among *Nothobranchius* populations are comparable with allopatric populations/cryptic species of Neotropical cichlids and much higher than in most African lake cichlids (Turner *et al.*, 2001; Ready *et al.*, 2006; Terzibasi *et al.*, 2008), making the *Nothobranchius* fishes interesting for comparative studies. Our current research is concentrated on molecular and behavioural studies of speciation in these fishes.

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