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Reproductive success of nuclear nonhybrid males of *Squalius alburnoides* hybridogenetic complex (Teleostei, Cyprinidae): an example of interplay between female choice and ecological pressures?

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Abstract The hybridogenetic fish complex *Squalius alburnoides* comprises diploid males with non-hybrid nuclear genomes and several hybrid forms varying in ploidy and relative proportions of the parental genomes. In this paper, we present evidence that in captivity females prefer to mate with non-hybrid males. We suggest that female choice combined with different ecological requirements of hybrid and non-hybrid males may explain the extreme variation in the relative abundance of male types among drainages.

Keywords Cyprinidae · Female choice · Sexual selection · Reproductive behaviour · Courting displays

Introduction

The *Squalius alburnoides* minnows are endemic to the Iberian Peninsula and constitute a complex of various ploidy forms, originated by interspecific hybridization between *S. pyrenaicus* females (P genome) and males from an extinct *Anaocypris*-like ancestor (A genome) (Alves et al. 2001; Robalo et al. 2006). Aside from variation in the

ploidy level, the *S. alburnoides* individuals also differ in the relative proportion of the P and A genomes (Alves et al. 2001). It is important to stress that both male and female diploid PA hybrids (the form that probably was at the origin of the complex) do not undergo meiosis, producing gametes clonally (reviewed in Alves et al. 2001). Most triploids are PAA females that reproduce by meiotic hybridogenesis once they exclude the P genome, after which the AA genomes undergo meiosis and recombination, generating haploid A gametes, although they can produce other gametes, namely, AA and even PAA eggs (Alves et al. 2004).

Meiosis is also apparently normal in PPAA tetraploids, a rare form that produces PA gametes (Alves et al. 2001). There is also a AA form, which is much smaller and morphologically distinct from the hybrid forms (Collares-Pereira 1984)—see details below. These diploid fish with nuclear non-hybrid genomes are males that undergo normal meiosis and generate A sperm. When the A sperm of these males meets A eggs from PAA females, new diploid males carrying the nuclear genome of the paternal ancestor are found again. This brief description outlines the high complexity and diversity of this fish complex, but we believe it is helpful to give some background on this unusual diploid–polyploid complex.

Although triploid females predominate in all populations, the frequency at which the other forms occur differs significantly among drainages—Table 1. In contrast with the situation in many other complexes of hybrid origin that are almost exclusively composed of females (Vrijenhoek et al. 1989), fertile males of different ploidies and genome constitutions are also found (Alves et al. 2001). Thus, females of *S. alburnoides* mate not only with males of other sympatric *Squalius* species—*S. carolitertii* in the northern rivers, *S. pyrenaicus* in the south and *S. aradensis* in the southwest—but also with males of *S. alburnoides* (Alves et al. 2001; Cunha et al. 2004; Pala and Coelho 2005; Sousa-Santos et al. 2005, 2006). The latter include not only individuals with hybrid nuclear genomes, but also non-hybrid diploids reconstituted from the hybrids and presenting the nuclear genome of the paternal ancestor in

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homozygosity (AA genome) (Alves et al. 2002). Their mitochondrial DNA is that of other *Squalius* species with which the complex hybridizes, the original mtDNA of the missing ancestor having been lost in evolution (Alves et al. 1997; Carmona et al. 1997).

The non-hybrid and hybrid males constitute two very distinct morphotypes, differing in size, morphology and ecological requirements (Collares-Pereira 1984; Martins et al. 1998; Ribeiro et al. 2003): non-hybrids are smaller than hybrids and the males of other sympatric *Squalius* species, and tend to prefer habitats characterized by shallow waters and higher temperatures, conditions that are typical of the rivers of south Iberia, when summer droughts reduce many streams to pools. In contrast, *S. alburnoides* females and other *Squalius* species show a preference for deeper waters and higher current velocities (Martins et al. 1998; Pires et al. 1999). The non-hybrid males are the most common male type in most of the southern populations but are apparently absent from the populations of the northern rivers (Table 1). The causes of these discrepancies among drainages are still unclear.

The studies of the processes of gamete formation in this fish complex (see details in Alves et al. 2001) support the conclusion that the persistence of these nuclear non-hybrid males is self-dependent, as they can only be generated from crosses involving males with their own genomic constitution and the most abundant form of triploid females (PAA). Their persistence in many populations constitutes a very interesting evolutionary problem, as the frequency of this type of males is inherently unstable. Indeed, if they are lost from a population, they are unlikely to be produced again. Furthermore, females do not depend on this male type, because they can mate with hybrid males and with the males of other *Squalius* species. Hence, some advantage must exist that explains the evolutionary persistence of this male type in the southern populations.

Female choice may play an important role in determining the persistence and abundance of the non-hybrid males in southern drainages: if, despite being smaller, the non-hybrid males are favoured by female choice this may explain their abundance in the southern drainages, where the ecological conditions tend also to favour them. To test this hypothesis, we investigated the spawning behaviour of *S. alburnoides* in

captivity in a setting where *S. alburnoides* females had free access to hybrid and non-hybrid conspecific males.

Materials and methods

A captive population of 56 *Squalius alburnoides* was established with individuals electrofished in the Tagus River basin (R. Sorraia), in the central part of Portugal. Fish were maintained in an outdoor aquarium (150×50×70 cm) under natural conditions of light and temperature, and were fed with commercial flake food. This density of fish that, at first glance, may look much higher than that found in nature, is likely not very different from the conditions that the complex meets in its natural habitats. Indeed, the complex lives for the most part in Mediterranean rivers and streams, which in the spring and summer get almost entirely reduced to small pools where fish are forced to congregate, being retained there for many months. This population showed breeding cycles that coincided with the reproduction in the field (see Ribeiro et al. 2003) and spawned spontaneously in 3 consecutive years.

In the absence of any externally visible sexual dimorphism, sex identification was made during the breeding season by applying a mild pressure on the abdomen of the fish. Mature males were easily identified by the release of milt ($N=11$), while in mature females a few eggs were extruded ($N=45$).

Three categories of fish were visually distinguished during the breeding season: females, nonhybrid males and hybrid males. Mature females were identifiable in the video recordings due to their larger size and swollen abdomens. The distinction between hybrid (mean body size of 83.35 ± 13.24 mm, $N=7$) and non-hybrid males (mean body size of 53.18 ± 9.56 mm, $N=4$) was based on morphological differences: non-hybrid males (previously described as “form B” of the complex by Collares-Pereira 1984) are small fish with a narrow body and pointed head, straight dorsal profile and convex ventral profile, terminal mouth with a greater lower jaw so that the opening is turned slightly upward, and deeply forked caudal lobes. The presence of a straightforward anterior portion of the lateral

Table 1 Minimum and maximum relative frequencies of the different forms of both genders of the *S. alburnoides* found in the northern and southern river basins (adapted from Alves et al. 2001 and Pala and Coelho 2005)

	Ploidy	Northern river basins			Southern river basins			
		Genome	Douro (%)	Mondego (%)	Genome	Tejo (%)	Sado ^a (%)	Guadiana ^a (%)
Females	2n	CA	0–4	0–10	PA	0–15	36–77	0–35
	3n	CAA/CCA	36–90	80–90	PAA/PPA	50–100	19–70	11–88
	4n	CCAA	0–1	–	PPAA	0–10	0–2	–
Males	2n	CA	10–14	5–14.9	PA	0–23	–	–
	3n	CAA/CCA	0–1	5.3	PAA/PPA	0–22	0–4	0–5
	4n	CCAA	0–1	0–5	PPAA	0–14	0–2	–
	2n	AA	–	–	AA	0–16	0–48	8–89

^aRiver basins subjected to extreme summer droughts

line was also an unambiguous diagnostic character used to discriminate between non-hybrid and hybrid males (which presented a typical curvilinear lateral line) (C. Sousa-Santos, unpublished data). This distinction was made after the observation of 33 specimens, whose homozygous genomic constitution was confirmed with the sequencing of the beta-actin nuclear gene (unpublished data, Sousa-Santos et al. 2005).

Ad libitum observations (sensu Martin and Bateson 1993) were conducted 5 days a week between March 18 and July 4, 2003. The breeding season extended from April 30 to July 3 during which 8 days of spawning activity were observed, comprising a total of 211 spawning events (with egg-laying). Spawning behaviour started early in the morning and continued until sundown. Maximum water temperatures, recorded daily during the observation period, ranged from 18 to 28°C (mean±SE=21.6±2.73°C). A total of 1,190 min of videotape recordings (Canon MV3 camera) were used to allow subsequent analysis of courtship and spawning. Agonistic behaviours, when present, were recorded. From all the spawning events recorded, 31 were chosen for the analysis due to optimal image requisites, such as the possibility to track the courting behaviours before the spawning and a clear distinction of the individuals involved. The number of times the focal females interrupted the spawning sequence when courted in different courtship contexts was compared with a Kruskal–Wallis test as implemented in Statistica 5.0 (Statsoft). Dunn's tests were subsequently performed to identify differences between specific contexts after the implementation of Siegel and Castellan (1988). To test if there was any bias favouring a given male type, we compared the frequencies of successful spawnings involving pairs with a hybrid and pairs with a non-hybrid male, against the frequencies that would be expected if females paired randomly. This null hypothesis assumed that the spawning pairs involving a hybrid or a non-hybrid would reflect the proportions of the two male types in the observation tank. This comparison was performed with a goodness of fit Chi²-square (χ^2), the significance of which was accessed by a simulation procedure with the program ADERSIM (Vitor Almada). This program generates 1,000 simulations in which values are randomly assigned to the different classes with probabilities reflecting their expected frequencies. The number of times out of 1,000 that for each class-observed values are equal or greater and equal or smaller than the simulated values allows the assessment of the significance of the results. In addition, the number of times that the χ^2 was equal or exceeded by the χ^2 computed for each simulation is also provided. The procedure is described in detail in Almada and Oliveira (1997) and has the advantage over the conventional χ^2 tests of being free of the assumptions of the χ^2 distributions, at the same time allowing the assessment of the significance of the deviations of individual classes.

Ethical note

Fishes were captured in a non-imperiled population. The sample size was chosen to maximize the likelihood of collecting all the forms in the complex but without risking depletion of the natural stock. Electrofishing was performed in low duration pulses to avoid killing juveniles (400–500 V and 2–4 A). All fish survived the transport to the laboratory, using aerated containers. The captivity conditions were enriched to simulate natural conditions with respect to vegetation and substrate. The sex determination procedure was performed while the fish were captured briefly in a hand net, and this took only a few seconds. Fish were immediately returned to the water and no casualties or behavioural abnormalities were detected.

Results

During the breeding season, fish aggregated close to the substrate, in the most aerated zone of the tank. Indeed, when a blue dye was added to the water close to the outlet of the filter pump, it became apparent that the spawning area was the part of the substrate more directly exposed to the current. All spawning sequences were initiated when a female entered the spawning area, where one or more males were displaying courtship behaviours: quivering, shuffling the dorsal and pectoral fins, and assuming “head-down” postures with the abdomen oriented towards the female. These male displays could evoke two different responses in females: 1) the female left the spawning site (and was generally chased by one or two courting males), and 2) the female also exhibited courting behaviours that culminated in the deposition of eggs. More details on the interactions between the two sexes during reproduction are provided in the “[Electronic supplementary material](#)”.

The spawning sequence was performed in synchrony by the female and by only one male in 61.29% ($N=19$) of the events, while the remaining spawnings involved trios (38.71%, $N=12$). When the female spawned in a trio, each male assumed a side by side position on one of the flanks of the female. Trios comprised two non-hybrid males ($N=5$) or one non-hybrid and one hybrid male ($N=7$). In pairs, when the female spawned with a single male, the male was a hybrid in only two out of 19 occasions and a non-hybrid in the remaining 17 cases. This difference was significant ($X_1^2=5.48$, $N=2$, $P<0.05$), allowing us to reject the null hypothesis that all males had equal chances to participate in spawnings. In all cases, it was the female that approached the spawning site, reaching to a very close proximity of the displaying male(s).

Concerning courting trios with two non-hybrid males ($N=5$), the first male manoeuvred to position himself side by side with the female, followed by a second male that was in the vicinity and occupied the vacant flank. Immediately thereafter, the typical spawning sequence was initiated. In trios with hybrid and non-hybrid males ($N=7$), the mate that first took the side-by-side position against the female was the non-hybrid male in three cases

and the hybrid male in the remaining events. However, it is interesting to note that when the hybrid male was the first to contact the flank of the female, the female initiated a series of manoeuvres and changes of position, which ceased only when the non-hybrid male occupied the vacant flank. At this stage, the female initiated a new spawning sequence, culminating with egg deposition. Before that, when only the hybrid male was present, the female always interrupted the sequence at an initial stage, manoeuvring to another place in the spawning site where the spawning sequence was initiated again.

We assumed that the number of times that the female interrupts a spawning sequence (IS value) is inversely related to its readiness to spawn. The medians of the IS value (and the interquartile ranges—IQR) for pairs with one non-hybrid, pairs with one hybrid, trios with non-hybrids and trios with one hybrid and one non-hybrid were 1 (IQR between 1 and 1), 1.5 (IQR between 1.25 and 1.75), 2 (IQR between 2 and 2) and 5 (IQR between 3 and 5.5), respectively. The medians of the four groups differed significantly (Kruskal–Wallis ANOVA on ranks: $H_{3, 31}=9.619$, $P=0.02$)—and all post hoc comparisons using a Dunn’s test were significant at $P<0.05$ (two-tailed test). The smallest values were found when the females were mating with a single non-hybrid male and were largest when a hybrid male was present in a trio.

During the spawning events, agonistic male–male interactions were negligible: two isolated agonistic behaviours were performed in the same spawning event by a hybrid towards a non-hybrid male (“pushing” and “blocking path”) and in two other different events, a hybrid male tried to position himself side by side with the female, laterally displacing the other male (a non-hybrid male in one of the events and a hybrid in the other).

Discussion

During the breeding season, *S. alburnoides* males gather in the spawning area and perform a series of courtship displays that attract females, which ultimately have the choice of the mate(s) and of the location and timing of the spawning. In fact, as described by Katano and Hakoyama (1997), the presence of courtship behaviour in fishes classified as open substratum lithophil spawners (Balon 1975), such as *S. alburnoides*, suggests that female mate choice likely plays an important role in determining male mating success.

Our data point to the existence of a female preference to spawn with nuclear non-hybrid males: 1) the majority of the spawning events involved at least one non-hybrid male, although there were more hybrid than non-hybrid males available; 2) there were significantly fewer interruptions by the female after the spawning sequence had been initiated when the female spawned with one non-hybrid male, than when the spawning event involved a trio with one non-hybrid and one hybrid male; and 3) in trios with one hybrid and one non-hybrid male, the female, when approached first by the hybrid male, manoeuvred to avoid him and only performed the complete spawning sequence when the non-hybrid male joined the pair. As the fish were not

individually marked, some degree of pseudoreplication that could limit the validity of our conclusions cannot be ruled out. Even if the fish were marked, with the large number of individuals involved and the speed of spawning movements, it would be very difficult to identify the participants reliably.

We believe, however, that the results presented are not artefacts due to mere idiosyncratic peculiarities of some individuals. In females, the abdomen shrinks after spawning and only gets swollen again after many days or weeks, when a female is ready to release a new batch of eggs (see Ribeiro et al. 2003). This difference between females that are ready to spawn and those that have just spawned made us almost sure that successive observations very likely represented spawning events involving different females. The males in our study differed in size and morphology, and, although we cannot demonstrate that the same male was not observed more than once, we are confident that the observations used here involved different individuals.

As non-hybrid males are much smaller than hybrid males and males of other *Squalius* species with which *S. alburnoides* females mate, it is not possible to test experimentally if the females are choosing based on size or on other attributes of males. Also, there are no interspecific studies on the choice by females in this group of *Squalius* and it is unknown how the differences in size may affect male success and female preferences.

Regardless of the mechanism involved in female choice, the important point is that non-hybrid males are favoured to the detriment of hybrid males, playing an important role in the reproduction of the *S. alburnoides* complex. This is surprising when we consider their small size and the fact that females may also mate with other males, both hybrid and members of other *Squalius* species. In fact, the smaller size of these nonhybrid males, which at first sight could be seen as a disadvantage, probably enables them to perform a more vigorous body quivering and faster manoeuvres. Their displays in a situation of high density of males can, therefore, represent more intense stimuli than the ones performed by other males, which could be a possible explanation for the preference of the females.

A similar situation was described for *Rhodeus sericeus*: the vigour of male courtship has the stronger effect on female mate choice decisions, followed by male body size, in experiments with no male–male competition (Reichard et al. 2005), despite the fact that in this species males actively defend territories (Smith et al. 2004), a condition that usually favours bigger males. This is in agreement with Andersson (1994) who compared several taxa and showed that large body size in males is usually selected via male–male competition and less often by female choice. There are no interspecific studies on mate choice by females in this group of *Squalius*.

As stated above, genetic evidence showed that the non-hybrid males are always sons of males of their own type, thus, their substantial frequency in southern drainages is in itself a direct proof that they achieve a substantial number of fertilizations. Given the preference of females for the small non-hybrid males, how can the difference in their

frequencies among drainages be explained? In the first place, as noted above, the mechanism of their production implies that if they became extinct from a population, they are unlikely to be regenerated. If ecological conditions are adverse to the non-hybrid males, as is probably the case in the north, where rivers have no summer droughts and tend to have stronger currents, they may be at a disadvantage when compared with hybrid males and males of other sympatric *Squalius*. Indeed, non-hybrids are so much smaller that it is unlikely that they are in a disadvantage when compared to larger males in the northern rivers, where currents are often strong. This may have led to a gradual decrease in their number even if the females from the northern populations had the same mating preferences for smaller non-hybrid males.

By contrast, in the south, their small size and tolerance to shallow, warm and still waters (Martins et al. 1998) combined with the female preference may help to maintain them in populations. In addition, the fact that the non-hybrids undergo normal meiosis that is absent in hybrids, which are mostly diploids that produce sperm clonally (Alves et al. 1999; Pala and Coelho 2005), will also contribute to maintain a higher level of genetic variability in the progeny. This fact may be important in the harsh and highly unpredictable Mediterranean environments of the south (Mesquita et al. 2005). Thus, the preference of the females for the small AA males may be evolutionarily advantageous due to the fact that these small males undergo normal meiosis and recombination, and females mating preferentially with them could benefit their offspring.

If our hypothesis proves to be correct, the persistence of non-hybrid males may depend upon a balance between the behavioural preferences of the females and differential ecological requirements of both male types, with distinct outcomes in different environments. Experimental tests in tanks simulating various habitats differing in water flow intensity, combined with DNA fingerprinting of adults and progenies, may provide a way to test these hypotheses. All these considerations assumed that the AA males, like all *S. alburnoides*, originated in the southern drainages, having subsequently migrated to the northern rivers (Alves et al. 1997). In this scenario, AA males should be present in *S. alburnoides* populations, as their origin and their absence in the north should be viewed as a secondary loss once it is difficult to explain why, during the process of colonization of northern rivers, the small AA males so common in several southern populations would be excluded from the northward dispersal event.

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