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Mestrado em Biologia da Conservação
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Abstract

The hybridogenetic complex *Squalius alburnoides* is constituted by several forms with different ploidies and combinations of parental genomes. Very little was known about the reproductive behavior of this cyprinid fish, which might play a key role in its dynamics' comprehension. Thus, the main goal of the present work was the study of reproductive behavior within the complex and also from an interspecific perspective. For the purpose, initially, a non-intrusive individual recognition system based on scale patterns was developed. Later, the intersexual selection of the most common *S. alburnoides* form (triploid females) was studied in northern populations. A reproduction tendency with individuals of the sympatric species *S. carolitertii* was observed. This preference pattern may give some important clues for clarifying *S. alburnoides* population and genetic dynamics. Hereupon, the present study evidenced the high importance of reproductive behavior to the comprehension of the dynamics of non-sexual complexes.

Keywords: Reproductive behavior, individual recognition, intersexual selection, hybridogenetic complex, *Squalius alburnoides*

Resumo

O complexo ciprinícola *Squalius alburnoides* resultou de um processo de hibridação unidireccional envolvendo fêmeas de *S. pyrenaicus* (genoma P) e machos de uma espécie próxima de *Anaecypris hispanica* (genoma A) provavelmente extinta. Tratando-se de um complexo, *S. alburnoides* é composto por várias formas, apresentando ploidias (2n=50; 3n=75 e 4n=100) e constituições genómicas distintas com diferentes proporções dos respectivos genomas parentais. A introgressão de ADN nuclear das espécies de *Squalius* simpátricas no complexo *S. alburnoides* é bastante comum, levando à substituição do seu genoma materno pelo genoma da espécie de *Squalius* presente na respectiva bacia hidrográfica e, consequentemente, à existência de várias combinações genómicas. No entanto, a introgressão de ADN mitocondrial é menos frequente, ocorrendo sobretudo nas bacias hidrográficas meridionais. Os fundamentos destes elevados níveis de introgressão de ADN mitocondrial, embora pontuais, não estão esclarecidos, podendo dever-se a diferentes origens do complexo ou a acasalamentos interespecíficos actuais envolvendo fêmeas das espécies de *Squalius*

simpátricas e machos de *S. alburnoides*. Já a introgressão de ADN nuclear deverá ter resultado unicamente de acasalamentos interespecíficos envolvendo, maioritariamente, machos das espécies simpátricas e fêmeas de *S. alburnoides*.

Este complexo hibridogenético apresenta diferentes modos de reprodução, os quais produzem gâmetas com genomas e mecanismos de transmissão/hereditariedade distintos. Deste modo, conhecendo o modo de reprodução de cada forma é, actualmente, possível prever a ploidia e a constituição genómica da descendência produzida por qualquer tipo de cruzamento. Embora o conhecimento do modo de reprodução adoptado por cada forma de *S. alburnoides* sugira uma constituição populacional teórica baseada na estocasticidade dos diferentes cruzamentos possíveis, a composição das populações naturais, para além de variar com a bacia hidrográfica, é discrepante do previsto no plano teórico, enfraquecendo o pressuposto de panmixia. Tais factos sugerem a existência de estratégias de selecção sexual que poderão, então, favorecer a manutenção de determinadas formas nas populações naturais em detrimento de outras. Vários estudos têm demonstrado que a selecção intersexual se apresenta relacionada com a viabilidade e sobrevivência da descendência, sendo esta melhor sucedida quando provém de acasalamentos com os parceiros sexuais escolhidos. Deste modo, a compreensão da dinâmica populacional de *S. alburnoides* poderá estar dependente de características do seu comportamento reprodutor, nomeadamente, da selecção intersexual, que pode, ainda, variar entre formas. Embora a composição das populações naturais varie grandemente entre bacias hidrográficas e até mesmo entre cursos de água da mesma bacia, as fêmeas triplóides dominam a maioria das populações, pelo que deverão ser de grande relevância na sua estruturação.

Assim, tendo em conta todos os aspectos descritos anteriormente, o presente trabalho teve como objectivo principal o estudo da selecção intersexual desempenhada pela forma de fêmeas mais comuns deste complexo hibridogenético nas populações do Norte de Portugal. Dado que as espécies de *Squalius* simpátricas parecem estar grandemente envolvidas nas dinâmicas genética e populacional do complexo, a selecção intersexual foi ainda abordada do ponto de vista interespecífico, tendo-se incluído no estudo a espécie simpátrica da região em estudo, i.e. *S. carolitertii*.

Em grande parte dos estudos de comportamento animal, o reconhecimento individual dos espécimes em análise é de extrema importância. De um modo geral, o uso de marcas artificiais é desaconselhado, uma vez que, para além de estar associado a um certo grau de desconforto e mortalidade, o seu efeito no comportamento dos indivíduos é normalmente imprevisível, sendo recomendado o uso de marcas naturais. As diferentes formas de *S. alburnoides* são morfologicamente bastante semelhantes (com algumas excepções), sendo a sua maioria de difícil distinção com base em características fenotípicas. Para além disso, a distinção entre indivíduos é também complexa, uma vez que *S. alburnoides*, à primeira vista, não apresenta padrões fenotípicos evidentes como, por exemplo, padrões de coloração que poderiam facilitar o reconhecimento individual. Assim, o presente trabalho teve como objectivo inicial a criação de um método de reconhecimento individual em *S. alburnoides* baseado em marcas naturais. Embora os indivíduos apresentem, como referido, um padrão de coloração muito homogéneo e uniforme, o tom e o brilho das escamas constituintes das regiões laterais e dorsal variam entre si, criando uma linha de contraste entre as referidas regiões, cujo padrão se demonstrou variar entre indivíduos. Este padrão de escamas permitiu discriminar indivíduos de diferentes ploidias visualmente e através de *software* adequado, tendo-se ainda demonstrado a sua utilidade para identificar indivíduos a longo prazo, devido ao reduzido grau de alteração temporal do referido padrão.

Após a definição do método de reconhecimento individual descrito anteriormente, procedeu-se, então, ao estudo da selecção intersexual de *S. alburnoides* em espécimes capturados nas populações do Douro, do Mondego e do Guadiana. Para o efeito, fêmeas triplóides foram sujeitas a testes de escolha de parceiros, nos quais lhes foram apresentados simultaneamente indivíduos-estímulo de diferentes ploidias e constituições genómicas e, ainda, indivíduos de *S. carolitertii*. As preferências das fêmeas triplóides em teste foram, então, avaliadas de acordo com o tempo despendido pelas mesmas junto de cada indivíduo-estímulo. Os resultados obtidos sugeriram que as referidas fêmeas parecem mostrar uma tendência de reprodução com machos de *S. carolitertii*. Este padrão é igualmente corroborado pela sua preferência por indivíduos de maior tamanho corporal, uma vez que, de um modo geral, *S. carolitertii* apresenta maiores dimensões do que as várias formas de *S. alburnoides*. Para além disso, a tendência de reprodução sugerida é coerente com a segregação espacial típica das fêmeas triplóides e de *S. carolitertii*, uma vez que possuem, durante o período reprodutor, preferências idênticas em termos de habitat, aumentando a sua probabilidade de encontro.

A ocorrência de cruzamentos interespecíficos envolvendo fêmeas de *S. alburnoides* e machos de *S. carolitertii* tem consequências directas para as dinâmicas genética e populacional do complexo.

Relativamente à sua dinâmica genética, tais cruzamentos permitem explicar os elevados níveis de introgressão de ADN nuclear da referida espécie simpátrica no complexo, nas respectivas bacias hidrográficas. Para além disso, a ocorrência de reprodução com indivíduos de uma espécie bissexuada garante a entrada de novos traços genéticos no complexo, contribuindo para o aumento e manutenção da sua variabilidade genética e, consequentemente, para o seu sucesso evolutivo.

As interpretações populacionais da preferência observada estão dependentes do conhecimento do modo de reprodução das formas de *S. alburnoides* envolvidas nos cruzamentos em questão. Relativamente às populações setentrionais, pouco se sabe em relação aos mesmos, sendo os dados disponíveis insuficientes para generalizar acerca da do(s) tipo(s) de gâmetas produzidos pelas fêmeas triplóides. Assim, considerando todos os possíveis modos de reprodução já observados em particular nas populações meridionais, os resultados obtidos neste estudo juntamente com o conhecimento da composição actual das populações do Norte de Portugal sugerem que as fêmeas triplóides estudadas (CAA) produzem os seus gâmetas maioritariamente através de hibridogénese simples, i.e. excluindo o genoma heterospecífico de *S. carolitertii* (C) e transmitindo ambas as cópias do genoma ancestral paterno (AA). Deste modo, os cruzamentos interespecíficos envolvendo estas fêmeas e machos de *S. carolitertii* levarão à produção de descendência com constituição genómica semelhante à das suas progenitoras (i.e. CAA), justificando a elevada proporção desta forma e a reduzida ocorrência da forma diplóide (CA) nas populações setentrionais. Os restantes modos de reprodução descritos (hibridogése meiótica e produção de gâmetas não reduzidos) poderão ter uma menor incidência nestas populações quando comparadas com as meridionais, aspecto que será investigado oportunamente.

Posto isto, o presente trabalho demonstrou a elevada importância do estudo do comportamento reprodutor para a compreensão da dinâmica das populações de *S. alburnoides*, à semelhança do observado em outros complexos não-sexuados.

Palavras-chave: Comportamento reprodutor, reconhecimento individual, selecção intersexual, complexo hibridogenético, *Squalius alburnoides*

Contents

General Introduction

The cyprinid complex *Squalius alburnoides* was originated from an unidirectional hybridization process resulting from crosses between *S. pyrenaicus* females (P genome) and males from a presumably extinct *Anaecypris*-like species (A genome) (Alves *et al.*, 2001; Crespo-López *et al.*, 2006; Gromicho *et al.*, 2006; Robalo *et al*., 2006). *S. alburnoides* is constituted by several nuclear forms with distinct ploidies (2n=50, 3n=75 and 4n=75) and genomic constitutions with variable combinations of parental genomes (reviewed in Collares-Pereira & Coelho, *in press*).

The sympatric *Squalius* species contribute to *S. alburnoides* genetic dynamics, which is evidenced by the differential levels of nuclear and mitochondrial DNA introgressions of those species into the complex (see **Research Paper**). Such introgressions might be a result of interspecific crosses involving individuals from the complex and from the respective sympatric species. The differential levels of nuclear and mitochondrial DNA introgression suggest that the direction of such crosses is likely to vary between drainages: in the southern ones, these crosses seem to occur in both directions, while in northern populations they seem to occur mainly in one, i.e. between *S. alburnoides* females and *S. carolitertii* males (the sympatric *Squalius* species of the referred region). These differences in the direction of interspecific crosses between northern and southern populations may be explained by several behavioral factors. In one hand, they might be related with the particular reproductive ethology of the sympatric species, i.e. *S. carolitertii* females may be less available/successful in reproducing with *S. alburnoides* males, compared to the females of the other *Squalius* species. On the other hand, the referred differences in interspecific crosses' direction might be related to complex intrinsic ethological factors. For example, if non-hybrid males are the ones mostly involved in interspecific crosses, the lower levels of *S. carolitertii* mitochondrial DNA introgression should be related with their absence in northern drainages (Sousa-Santos *et al.*, 2007a).

S. alburnoides presents sexual and non-sexual reproductive modes which vary between forms (Alves *et al.*, 1998, 1999, 2001, 2004; Gromicho & Collares-Pereira, 2004; Pala & Coelho, 2005; Crespo-López *et al.*, 2006; Sousa-Santos *et al.*, 2007b). The knowledge of the reproductive mode(s) of each form allows predicting the genomic constitution of offspring produced by different types of crosses and, consequently, calculating the theoretical composition of a panmitic population. However, the composition of *S. alburnoides* natural populations differs drastically from theoretical predictions, thus crosses might not occur randomly in *S. alburnoides*, suggesting the existence of sexual selection.

Besides, *S. alburnoides* natural populations vary greatly between drainages and even between rivers (Alves *et al.*, 2001; Pala & Coelho, 2005; Cunha *et al.*, 2008). Such variations in forms' distribution suggest the existence of adaptive differences between forms, probably related with both parental genomes' expression in *S. alburnoides* phenotype (Pala *et al.*, 2008). The differential adaptations are well evidenced by the spatial segregation of habitat and feeding niches that were observed between forms (Martins *et al.*, 1998; Gomes-Ferreira *et al.*, 2005).

Considering the adaptive differences described above, it seems likely that *S. alburnoides* forms might also present different behavioral strategies, namely in reproductive behavior which, for the many reasons referred, seems to play a key role in its dynamics. However, very little is known about the reproductive behavior of *S. alburnoides*. A preliminary study in southern populations revealed some clues about male differential reproductive success (see **Research Paper**). The same study also evidenced the existence of a negative relation between male density and agonistic behaviors' frequency, suggesting the existence of alternative density-dependent behavioral tactics (Sousa-Santos, 2007). As observed in many fish species (Mills & Reynolds, 2003; Reichard *et al.*, 2004a, b), when population density is high, males might stop defending territories, since, due to the high intrusion pressure, the costs of such defense would overcome the benefits. Moreover, in general, smaller males are less effective in territorial defense (Candolin & Voigt, 2001; Mills & Reynolds, 2003), thus taking advantage of a high density scenario. Such patterns might, somehow, explain the fact that non-hybrid males (smaller than the hybrids) are only distributed in southern drainages, once, during summer, these basins are reduced to pools with high fish density, which does not occur in northern basins (Sousa-Santos, 2007). Therefore, *S. alburnoides* reproductive behavior might also help to explain the differential distribution of forms.

Hereupon, the reproductive behavior of *S. alburnoides* complex seems to be a keystone to the clarification of several aspects of its dynamics. Thus, the main goal of this thesis was the assessment of the reproductive behavior of the complex, namely, the study of its intersexual selection from a within-complex and an interspecific perspective.

The results obtained are here presented in two publications, one currently under review at *Journal of Fish Biology* as a “Brief Communication” and the other to be submitted later as a “Research Paper”. The first one describes a non-intrusive individual recognition method based in natural marks, namely, in scale patterns. The second one focuses on the intersexual selection of the most common female form of the complex, evidencing an apparent reproduction tendency with males of the sympatric species *S. carolitertii* over the ones from the complex.

This study, while raising new questions, allowed identifying some prospects for further research on the complex behavior strategies, which are presented at the end of the dissertation, due to their high relevance also within a conservation biology specific context.

Scaleprinting methodology for studying behaviour in fish: the case of *Squalius alburnoides* (Steind.) complex (Cyprinidae)

M. Morgado-Santos, I. Matos, L. Vicente and M. J. Collares-Pereira

Abstract: A nonintrusive method for individual identification of Iberian cyprinid complex *Squalius alburnoides* is presented, with possible application to other fish complexes and species. The proposed methodology is based on scale patterns that vary greatly between individuals.

Keywords: Individual recognition; natural marks; scale patterns; temporal validation

In most studies of animal behaviour, the easy individual identification of the specimens is of extreme importance. Though there are several tagging techniques that allow a straightforward individual recognition of the specimens, in a general way, the application of such tags is not recommended in studies of animal behaviour. Their effect on the subject's behaviour is almost always unpredictable (Burley, 2006), besides being associated with some degree of animal discomfort and mortality risk. Thus, in behavioural studies, individual recognition should be based on natural marks (Martin & Bateson, 2007).

The present study focuses on the finding of an individual identification system in a freshwater fish complex, *Squalius alburnoides* (Steind.), based on scale patterns that vary greatly between individuals. Such patterns are visible in the contrast line situated between the dorsal and lateral regions of this cyprinid fish, whose scales show a totally different tone and brightness (Figure 1).

S. alburnoides is a hybridogenetic complex that resulted from interspecific crosses between *S. pyrenaicus* (Günther) females (P genome) and males from a missing ancestor *Anaecypris hispanica* (Steind.)-like (A genome) (Alves *et al.*, 1997, 2001; Gromicho *et al.*, 2006; Robalo *et al.*, 2006). *S. alburnoides* is constituted by nuclear hybrid and non-hybrid forms with different ploidies (2n=50, 3n=75 and 4n=100, though dominated by triploid females) and distinct combinations of parental genomes (reviewed in Collares-Pereira & Coelho, *in press*). In this non-sexual complex, as in others, specimens are morphologically very similar and difficult to identify either by form or individually, constraining any type of behaviour studies, which are now considered essential to understand the reproductive success of the distinct biotypes and, consequently, the evolutionary dynamics of natural populations.

Thus, the meaning of the present study was the assessment of the individual identification success based on the referred scale patterns, regardless of the individual's size, ploidy and genomic constitution and of the drainage from where the subjects came. Moreover, it was also a goal to assess the changing of scale patterns over time. For the purpose, in total, the scale patterns of 54 individuals from different catchments were analyzed and specifically collected at Douro (*N*=10), Mondego (*N*=10), Tagus (*N*=15), Guadiana (*N*=9) and Almargem (*N*=10) (from North to South). Fish were captured by electrofishing, transported to the laboratory in aerated containers and kept in indoor aquariums in accordance with the recommended ethic guidelines (ASAB, 2006).

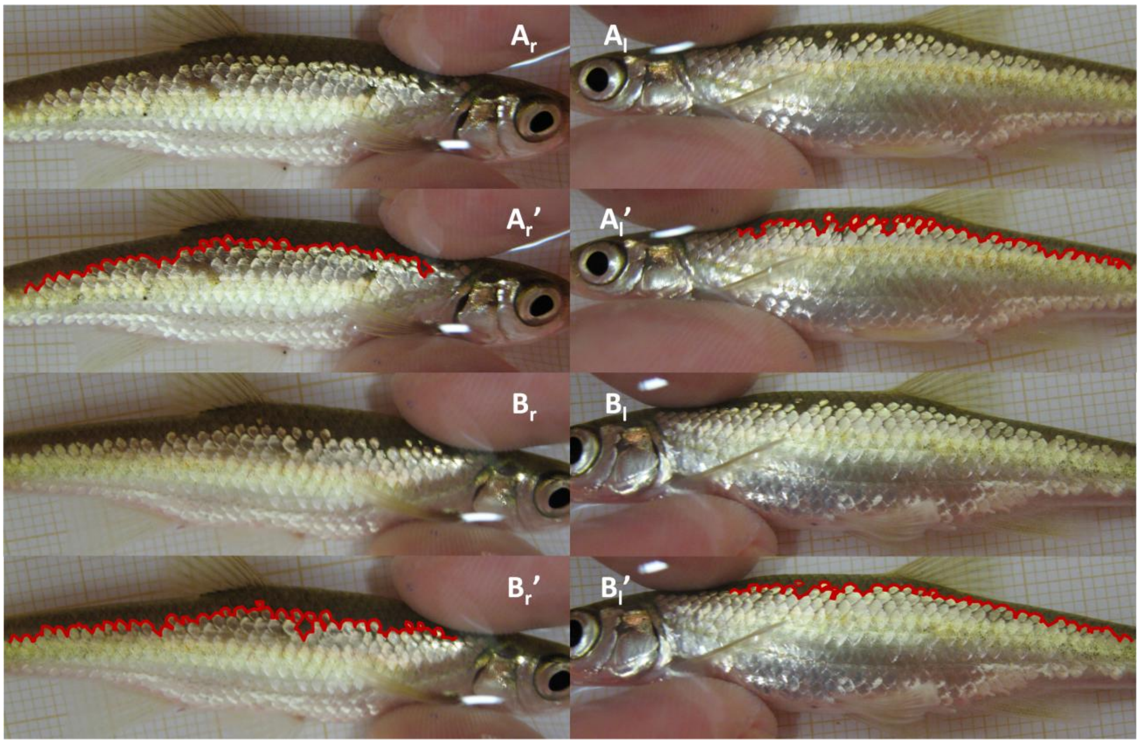

Figure 1. Contrast lines from both sides of two triploid individuals from Mondego drainage (A_r: right side of individual A; A_r': contrast line of the right side of individual A; A_l: left side of individual A; A_l': contrast line of the left side of individual A; B_r: right side of individual B; B_r': contrast line of the right side of individual B; B_l: left side of individual B; B_l': contrast line of the left side of individual B).

The validation of the individual identification method based on scale patterns was made with the help of another identification system, namely by marking, i.e. cutting of small fin portions in each subject. For this, specimens were anesthetized using a low concentration solution of tricaine mesylate (MS-222). After being unresponsive to tactile stimuli, fish were photographed by one of the authors (MMS) and fin clipped by another author (IM) to create a code that easily allowed the individual identification of the subjects. The photographs were taken from both sides of the fish, in a lateral position over a graph paper sheet immersed in a thin layer of water (about 2 cm deep). The individuals were maintained in different aquaria, in groups of five to 10, to allow the repetition of fin codes. The numbers attributed to each fish were the same for both identification codes (photographs and fin cuts), though each author had only access to his/her own code.

After this process, the ploidy of all individuals was determined through DNA content measurements of erythrocytes by flow cytometry, using a Coulter Epics XL cytometer. Following the method described in Próspero and Collares-Pereira (2000), all specimens were once again anesthetized and ventrally positioned. The syringe, containing about 50 µL of freezing solution (40 mM citric acid trisodium salt, 0.25 M sucrose and 5% dimethyl sulfoxide; pH=7.6), was inserted in the region posterior to the anal fin, pulling the plunger immediately after the insertion, thus creating vacuum in its interior. This vacuum allowed the immediate collection of blood once the caudal vein was hit, reducing the time of the procedure and avoiding the entrance of air and/or freezing solution to the blood strain of the fish. The amount of blood taken from each fish (approximately 50 µL) was stored in individual eppendorfs containing 500 µL of freezing solution and all samples were kept at -80ºC. The results from DNA flow cytometry showed, as expected, different proportions of each form depending on the specific drainage (Almargem – 40% 2n, 60% 3n; Douro – 20% 3n, 80% 4n; Guadiana – – 100% 3n; Mondego – 100% 3n and Tejo – 93% 3n, 7% 4n).

A day after photographing all specimens, each one was taken from the maintenance aquaria to be individually identified. Note that only IM knew the aquaria and, therefore, the drainage of each fish. MMS visually compared each individual to all photographs and was able to identify all the subjects correctly by their individual scaleprinting, confirmed by the code of fin cuts (IM code).

Around half of these specimens (29) were also previously photographed, namely the individuals from the drainages of the rivers Mondego (*N*=10; 11 January 2009), Douro (*N*=10; 22 March 2009) and Guadiana (*N*=9; 10 May 2009). The comparison of these photos with the ones taken in 28 and 29 July 2009, allowed the assessment of the identification method success over time.

First, all the 29 individuals were visually identified. In comparison to the identification made one day after the last photographs were taken, this matching was more difficult, since the scale patterns used had, in some fish, relatively changed. However, some parts of the referred pattern, whose probability of repetition between subjects was considered very low, were maintained, allowing the identification of all fish without problem. All the photographs were further analyzed using the software I^{3}S, Interactive Individual Identification System (Reijns & Hartog, 2007), thus comparing the photographs from both sides of the fish taken in 28 and 29 July 2009, with the older ones. In the referred software, the reference points used were the centre of the pupil, the insertion of the first dorsal fin ray and the insertion of the inferior caudal fin ray, then marking the scales of the lateral region of the fish that were in contact with the ones typical from the dorsal region (Figure 2). Although the analysis of the left side of the specimens matched the visual identification of all fish, the analysis of the right side was not concordant for all of them. However, in general, the scores obtained (with zero representing total matching) for the left side photographs were considerably lower when compared to the ones obtained for the right side photographs, being this difference in the scores between sides enhanced in the mismatching identifications of the right side photographs (Table I). Thus, considering only the side with lower scores, all the identifications made by the software were congruent with the visual identification. The differences found between the individual identification through right and left sides of the fish are of difficult explanation and could be just related to the photography technique (for example, different angles when taking right or left side photographs). Another possible explanation, although less probable, may lie in the fact that the right and left sides of the fish have different change rates of their scale pattern, which has no apparent biological sense. The average scores obtained for both sides of the subjects for all populations (Table I) were the expected, with the Mondego population, whose individuals were photographed in 11 January 2009, obtaining the highest values for both sides (0.343 and 0.565 for left and right sides, respectively). The exception was the Guadiana population which obtained a higher average score for individuals' left side (0.319) when compared to Douro population (0.264), although the photographs were more recent (10 May 2009 against 22 March 2009). In general, these average scores showed that the scale pattern of the fish changed through time, although not significantly, since the identification was possible for all specimens.

Table I. Results obtained from software I^{3}S, namely the scores for both sides of the fish and the respective side(s) matching the visual identification of the subjects.

Hydrographic basin (Photograph's date)	Ploidy	I^{3}S score for left side	I^{3}S score for right side	Matching side(s)
Guadiana (10 May 2009)	3n	0.233	0.200	L/R
	3n	0.310	0.252	L/R
	3n	0.341	0.308	L/R
	3n	0.265	0.459	L
	3n	0.343	0.178	L/R
	3n	0.453	0.476	L/R
	3n	0.401	0.404	L/R
	3n	0.237	0.483	L/R
	3n	0.289	0.407	L
	AVERAGE	0.319	0.352	-
Douro (22 March 2009)	4n	0.274	0.418	L/R
	3n	0.296	0.504	L/R
	3n	0.257	0.512	L
	4n	0.317	0.457	L
	4n	0.297	0.486	L
	4n	0.331	0.518	L
	4n	0.291	0.614	L
	4n	0.233	0.467	L
	4n	0.176	0.223	L/R
	4n	0.163	0.647	L/R
	AVERAGE	0.264	0.485	-
Mondego (11 January 2009)	3n	0.273	0.619	L/R
	3n	0.261	0.461	L/R
	3n	0.249	0.512	L/R
	3n	0.286	0.629	L
	3n	0.414	0.595	L
	3n	0.295	0.553	L/R
	3n	0.367	0.720	L
	3n	0.526	0.696	L/R
	3n	0.340	0.346	L/R
	3n	0.420	0.516	L/R
	AVERAGE	0.343	0.565	-

To conclude, the present work evidenced the existence of a very useful natural system for individual identification based on scale patterns, which can be used even in long run studies (at least lasting seven months). Indeed, this method allowed, with straightforwardness, the individual recognition of *S. alburnoides* specimens (any ploidy) of the major hydrographical basins. It can be considered a window-of-opportunity also for other fish species with such scale patterns and, consequently, very useful to several kinds of studies, namely those related to behaviour and reproductive performance requiring subjects' individual identification.

Figure 2. Marked scales from both sides of the same individual (shown in Figure 1 as individual B) in the software I^{3}S.

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How does the population dynamics of *Squalius alburnoides* complex reflect female mate choices? A new behavioral approach into the complex's comprehension

Abstract: The hybridogenetic complex *Squalius alburnoides* is constituted by several forms with different ploidies (2n, 3n and 4n) and genomic constitutions with distinct parental genomes' combinations. This cyprinid complex shows an intricate population dynamics, evidenced by differences found in natural populations' composition, with variable nuclear and mitochondrial DNA introgression levels of the sympatric *Squalius* species into the complex. As observed in other hybrid non-sexual complexes, the reproductive behavior of the distinct *S. alburnoides* forms may play a key role to the clarification of such dynamics. Thus, the present work studied the intersexual selection occurring in northern *S. alburnoides* populations, which are, in a general way, highly dominated by a particular type of triploid females with a central role in the complex maintenance. Their intersexual selection was assessed through mate choice tests, in which four types of *S. alburnoides* individuals and one *S. carolitertii* (sympatric *Squalius* bisexual species in northern populations) were simultaneously presented as stimulus individuals. With the aim of discriminating the type of stimuli used in individuals' recognition, several trial sets were conducted, in which the stimuli passage was manipulated. It was shown that the most common *S. alburnoides* female form is likely to have a preference for *S. carolitertii* males and for bigger individuals. Furthermore, mate recognition seems to occur only through multichannel communication, since females only showed a preference pattern when no restriction in stimuli passage was made. The obtained results were interpreted given the current composition of *S. alburnoides* populations, and the evolutionary trend of such populations predicted based on the mate preferences of the most common female form.

Keywords: Mate choice, interspecific crosses, hybridogenetic complex, *Squalius carolitertii*, Cyprinidae

Introduction

The *Squalius alburnoides* (Steind.) complex resulted from a unidirectional hybridization process involving *S. pyrenaicus* (Günther) females (P genome) and males from an *Anaecypris hispanica* (Steind.)-like probably extinct species (A genome) (Alves *et al.*, 2001; Crespo-López *et al.*, 2006; Gromicho *et al.*, 2006; Robalo *et al.*, 2006). Being a complex, *S. alburnoides* is constituded by males and females with different ploidies (2n=50, 3n=75 and 4n=100, though mainly dominated by triploid females) and genomic constitutions with distinct combinations of parental genomes (reviewed in Collares-Pereira & Coelho, *in press*).

Due to interspecific crosses involving, mainly, males from the sympatric *Squalius* species and *S. alburnoides* females, the introgression of these species' nuclear genomes into the complex is very common, leading to the total substitution of the maternal ancestor genome (P) and, consequently, to the existence of several genomic combinations which have differential distribution and variable sex ratios (Table I) (Alves *et al.*, 2001; Pala & Coelho, 2005; Sousa-Santos *et al.*, 2007a; Cunha *et al.*, 2008). However, mitochondrial DNA introgression is less frequent in the complex, occurring specially in southern Portuguese drainages (Sousa-Santos *et al.*, 2006a, 2007a). These mitochondrial DNA introgressions still lack a clear explanation, since they may have resulted either from multiple independent origins of the complex (Alves *et al.*, 1997, 2002; Cunha *et al.*, 2004) or from interspecific crosses involving females of the sympatric *Squalius* species and *S. alburnoides* males (Sousa-Santos *et al.*, 2007a).

S. alburnoides complex has sexual and non-sexual reproductive modes that vary between forms, producing gametes with different genomes (Table II) (Alves *et al.*, 1998, 1999, 2001, 2004; Gromicho & Collares-Pereira, 2004; Pala & Coelho, 2005; Crespo-López *et al.*, 2006; Sousa-Santos *et al.*, 2007b). Alongside with the sympatric *Squalius* species, the only forms that contribute to increase complex's genetic variability are the ones that present sexual reproductive modes (with meiotic recombination). Thus, offspring success may, somehow, be higher in such forms. Therefore, it is easy to accept that *S. alburnoides* may have evolved advanced sexual selection tactics that, allowing the choice of particular forms as sexual partners, help to increase and maintain the genetic variability within the complex (Brown, 1997).

The current knowledge of the reproductive modes of each *S. alburnoides* form allows calculating the composition of a theoretical population based on the panmixia principle. However, natural populations' compositions (Table I) is drastically distinct from the one that theory predicts, thus suggesting that mates may not occur randomly.

Once both *S. alburnoides* parental genomes are expressed (Pala *et al.*, 2008), the various forms differ in several phenotypic traits, thus each one should show particular adaptations, leading to a decrease in niches' overlap. The referred distinct adaptations are evident in habitat and feeding niche spatial segregation observed between *S. alburnoides* forms, especially in drought periods which are characterized by low prey availability (Martins *et al.*, 1998; Gomes-Ferreira *et al.*, 2005). Actually, such adaptive differences could lead to the observed variations in *S. alburnoides* populations' composition along their distribution range (Table I). The phenotypic differences found between some forms are quite obvious in morphological traits, since the various *S. alburnoides* types form a continuum, suggesting the existence of a dosage effect in terms of genetic expression in hybrid phenotypes (Cunha *et al.*, 2009; Sousa-Santos *et al.*, unpublished). Furthermore, the phenotypic differences between *S. alburnoides* forms may directly influence sexual selection. In one hand, females might prefer some particular male morphological traits. On the other hand, females' mate choice could be conditioned by male differential availability, resulting from the spatial segregation described above. Moreover, *S. alburnoides* forms might have distinct behavioral traits, namely in reproductive behavior, which may be reflected in their sexual selection strategies.

However, very little is known about *S. alburnoides* reproductive behavior. A preliminary study by Sousa-Santos *et al.* (2006b) with specimens from southern populations revealed that males concentrate in spawning areas, adequate for egg development, to court females attracted by the high male density. As observed in other fish with external fertilization, the entrance of a female in the spawning area is, in general, followed by brief defense episodes and male chasing behaviors trying to reach females quickly than their competitors. Females seem to make the final decision about the specific spawning site and mate, by evaluating males' exhibitions (Sousa-Santos *et al.*, 2006b). In fact, although *S. alburnoides* has been eco-ethologically classified as an open substratum lithophil spawner (gamete release and abandonment occurring in unprepared substratum; Balon, 1975), courtship behaviors were observed (Sousa-Santos *et al.*, 2006b), suggesting the occurrence of mate choice (Katano & Hakoyama, 1997).

The same preliminary study evidenced that, in a high density scenario, non-hybrid males (AA genome) have higher reproductive success compared with the hybrid ones. It was identically demonstrated that agonistic behaviors' frequency increases considerably outside the spawning area, where male density is much lower (Sousa-Santos, 2007). These results suggest that, similarly to what happens in other species (Mills & Reynolds, 2003; Reichard *et al.*, 2004a, b), different forms of *S. alburnoides* males may present density-dependent alternative behavioral tactics (Sousa-Santos, 2007).

Table I. Ploidies, genomic constitutions and sex from *S. alburnoides* natural populations of the major river basins (Alves *et al.*, 2001; Pala & Coelho, 2005; Sousa-Santos *et al.*, 2007a; Cunha *et al.*, 2008). The variation ranges of relative frequencies are based on the results obtained from several samples collected in the respective river basins.

Drainage		Sympatric *Squalius* species	Ploidy and genomic constitution			
			2n=50		3n=75	4n=100
North	Douro	*Squalius carolitertii* (C genome)	AA ♀: 0% ♂: 0%	CA ♀: 0 - 4% ♂: 0 - 14%	CAA and CCA ♀: 3 - 90% ♂: 0 - 8%	CAAA, CCAA and CCCA ♀: 0 - 52% ♂: 0 - 50%
	Mondego	*Squalius carolitertii* (C genome)	AA ♀: 0% ♂: 0%	CA ♀: 0 - 10% ♂: 5 - 15%	CAA and CCA ♀: 36 - 90% ♂: 0 - 5%	CAAA, CCAA and CCCA ♀: 0% ♂: 0 - 5%
South	Tagus	*Squalius pyrenaicus* (P genome)	AA ♀: 0% ♂: 0 - 16%	PA ♀: 0 - 15% ♂: 0 - 23%	PAA and PPA ♀: 50 - 100% ♂: 0 - 22%	PAAA, PPAA and PPPA ♀: 0 - 10% ♂: 0 - 14%
	Sado	*Squalius pyrenaicus* (P genome)	AA ♀: 0% ♂: 0 - 48%	PA ♀: 36 - 77% ♂: 0%	PAA and PPA ♀: 19 - 70% ♂: 0 - 4%	PAAA, PPAA and PPPA ♀: 0 - 2% ♂: 0 - 2%
	Guadiana	*Squalius pyrenaicus* (P genome)	AA ♀: 0% ♂: 8 - 89%	PA ♀: 0 - 35% ♂: 0%	PAA and PPA ♀: 11 - 88% ♂: 0 - 5%	PAAA, PPAA and PPPA ♀: 0% ♂: 0%
	Quarteira	*Squalius aradensis* (Q genome)	AA ♀: 0% ♂: 45 - 100%	QA N/D: 0% - 5%	QAA and QQA N/D: 0% - 45%	QAAA, QQAA and QQQA N/D: 0% - 5%

However, in that work, no distinction between hybrid males and females was made. Considering the differences in phenotypic traits and in reproductive modes between forms that, consequently, lead to the production of distinct offspring forms, it is extremely important that sexual selection studies include a high number of forms and both genders. Besides, the mentioned study did not include individuals of the sympatric *Squalius* species that seem to play a key role in population and genetic dynamics of *S. alburnoides*. Moreover, no distinction was made between inter and intrasexual selection, whose isolated effects could be distinct (Qvarnström & Forsgren, 1998; Reichard *et al.*, 2005; Casalini *et al.*, 2009), especially because *S. alburnoides* males seem to present alternative behavioral tactics according to extrinsic conditions, namely, density (Sousa-Santos, 2007).

Table II. Expected reproductive modes, ploidies and genomic constitutions of offspring of hypothetical crosses between all known *S. alburnoides* forms, in which the S genome corresponds to the respective sympatric *Squalius* species (based on data from Alves *et al.*, 1998, 1999, 2001, 2004; Gromicho & Collares-Pereira, 2004; Crespo-López *et al.*, 2006; Sousa-Santos *et al.*, 2007b). Non-viable offspring (NV) are characterized by a ploidy level higher than 4n. Asymmetrical tetraploids (SSSA and SAAA), apparently more rare, were not included since their reproductive modes remain unknown. The spermatogenesis of SSA males is also unknown, though assumed to be similar to the one of SAA males. Legend: CP – Clonal production; H – Hybridogenesis; MH – Meiotic hybridogenesis; NM – Normal meiosis; NV – Not viable.

				Males					
			Genomic constitution	AA	SA	SAA	SSA	SSAA	SS
			Reproductive mode	NM	CP	CP	CP	NM	NM
Females	Genomic constitution	Reproductive mode	Gametes	a	sa	saa	ssa	sa	s
	SA	CP	sa	SAA	SSAA	NV	NV	SSAA	SSA
	SAA	MH	a	AA	SAA	NV	NV	SAA	SA
		H	aa	AAA	SAAA	NV	NV	SAAA	SAA
		CP	saa	SAAA	NV	NV	NV	NV	SSAA
	SSA	MH	s	SA	SSA	SSAA	SSSA	SSA	SS
	SSAA	NM	sa	SAA	SSAA	NV	NV	SSAA	SSA
	SS	NM	s	SA	SSA	SSAA	SSSA	SSA	SS

A recent study revealed that offspring success seem to be more related with female mate choices than with male dominance and competition, since offspring produced by crosses between females and their preferred males, regardless of intrasexual selection, showed higher levels of survival (Casalini *et al.*, 2009). Other studies also showed that females could take advantage in mating with non-dominant males (Jacob *et al.*, 2007; Reichard *et al.*, 2007).

Therefore, the principal goal of the present work was the study of *S. alburnoides* intersexual selection by assessing the mate choices of the most common female form in northern Portuguese drainages, choosing between several *S. alburnoides* forms and also *S. carolitertii* (Doadrio) (the sympatric bisexual species). Thus, its main objective was to determine which crosses might have a higher probability of occurrence and consequently of producing, theoretically, more successful offspring in such populations. This approach was expected to give some important clues for the comprehension of *S. alburnoides* population dynamics.

Materials and methods

Individuals capture, transportation and maintenance

The individuals used were captured by electrofishing, with a variable voltage (300-600 V) depending on the river conductivity, to maximize capture effectiveness. The pulses used were of low duration to avoid killing juveniles. In each electrofishing episode, 80 m of each river was sampled and, since *S. alburnoides* forms show spatial segregation, sampling covered different habitat types, varying in current velocity, vegetation and substrate.

Fish were captured in three rivers, each one located in a distinct drainage, namely, from North to South, in Rivers Paiva (Douro drainage; N=15; captured in 21 April 2009), Alva (Mondego drainage; N=11; captured in 12 November 2008) and Murtega (Guadiana drainage; N=7; captured in 19 April 2009). *S. carolitertii* individuals used in this study (N=3) were captured in River Paiva. Thus, a total of 30 *S. alburnoides* and 3 *S. carolitertii* specimens were collected and used in this work in accordance with the recommended ethic guidelines (ASAB, 2006). Note that, since determination of ploidy and genomic constitution of *S. alburnoides* individuals is only possible in laboratory, not all fish sampled were used in the behavioral tests. The numbers previously recorded only refer to the individuals tested.

Fish were transported to the laboratory in aerated containers, all having survived the transportation. In laboratory, individuals captured before the breeding season (Mondego drainage) were kept at room temperature and 12 hour light photoperiod, in a 120 L aquarium (100×30×40 cm). After collecting River Paiva individuals (Douro drainage), all individuals (from Mondego and Douro) were kept together in a 240 L aquarium (150×40×40 cm) at 22º water temperature and 14 hour light photoperiod, thus trying to induce fish maturation. About one week later, almost all fish matured and, then, the maintenance aquarium was equally divided in two parts with a perforated transparent acrylic plate (holes 3 cm apart from each other and with 4 mm diameter; 169 holes in total) to separate males from females, thus trying to prevent spawning occurrence, but allowing the passage of visual, chemical and acoustical stimuli between them. Following the method described in Alves *et al.* (2004), the sex of mature individuals was determined by applying a slight pressure in their abdomens, thus releasing a small quantity of oocytes or sperm. Since some *S. alburnoides* forms present a slight sexual dimorphism in body dimensions (females bigger than males), immature individuals were separated according to that characteristic and their gender confirmed after maturation. In what concerns individuals that never matured, sex confirmation was impossible, therefore such individuals were killed after the behavioral trials and their gonads observed under microscope to determine gender. With totally different genomic constitutions from Mondego and Douro individuals (Table I), specimens from River Murtega (Guadiana drainage) were maintained in a different aquarium with 160 L (100×40×40 cm), at the same water temperature and photoperiod conditions described above. The individual recognition of all fish used in this study was made following the nonintrusive method described in Morgado-Santos *et al.* (under review).

All individuals were fed twice a day, namely with commercial flakes for freshwater fish in the morning and with frozen brine shrimp (*Artemia salina*) in the afternoon. The maintenance aquaria were equipped with bottom filters and with substrate and rocks, thus mimicking the fish natural habitat.

Ploidy and genomic constitution assessment

Following the method described in Próspero and Collares-Pereira (2000), the ploidy of all individuals was determined through DNA content measurements of erythrocytes by flow cytometry, using a Coulter Epics XL cytometer. All specimens were anesthetized using a low concentration solution of tricaine mesylate (MS-222) and ventrally positioned after being unresponsive to tactile stimuli. The syringe, containing about 50 µL of freezing solution (40 mM citric acid trisodium salt, 0.25 M sucrose and 5% dimethyl sulfoxide; pH=7.6), was inserted in the region posterior to the anal fin, pulling the plunger immediately after the insertion, thus creating vacuum in its interior. This vacuum allowed the immediate collection of blood once the caudal vein was hit, reducing the time of the procedure and avoiding the entrance of air and/or freezing solution to the blood strain of the fish. The amount of blood taken from each fish (approximately 50 µL) was stored in individual eppendorfs containing 500 µL of freezing solution and all samples were kept at -80ºC.

After blood collection, fish returned to the maintenance aquaria and recovered from the anaesthesia just after. To avoid possible infections in the insertion area of the syringe, the water of the aquaria was treated with antibiotic (commercialized as Myxazin from Waterlife trademark) following the manufacturer instructions.

After ploidy assessment, triploid individuals were genotyped to determine their genomic constitution (CAA or CCA). All other individuals were not genotyped since their genomic constitutions were predictable based on ploidy results, morphological traits and/or population composition of the specific sampling locations based on previous studies. So, a) *S. carolitertii* individuals were morphologically identified; b) *S. alburnoides* diploid individuals were easily confirmed as AA based on morphological traits, and c) *S. alburnoides* tetraploid individuals were all considered as balanced (CCAA), since the asymmetric ones (CAAA and CCCA) were never found in natural populations (Cunha *et al.*, 2008).

DNA extraction followed the procedure described in Miller *et al.* (1988) and genomic constitutions were assessed following the method described in Sousa-Santos *et al.* (2005).

Behavioral tests

Mate choice trials took place in a tank specifically designed for the purpose (Figure 1) (adapted from Schlupp *et al.*, 1994), which was surrounded by a white curtain, thus preventing any external perturbation during experiments. The behavioral trials were recorded using a JVC digital camera placed above the referred aquarium. To allow the capture of the entire test aquarium in the recordings, a Kenko wide lens (0.5×) was used in the camera.

According to the results obtained from the ploidy and genomic constitution assessment, 11 triploid CAA females (from Mondego drainage) were tested for their mate preferences. For this, five different stimulus individuals were simultaneously presented to each female in test, namely, one *S. carolitertii* (CC), three *S. alburnoides* (AA, CCA and CCAA) and, as a control, one *S. alburnoides* female (CAA from the same sampling collection of the tested females). Note that the distribution area of non--hybrid *S. alburnoides* males (AA) does not include the river from where the tested females came from (Mondego), being totally naive to these stimulus males. The aim of including this male type in the trials was the assessment of their theoretical higher reproductive success related to female preference (Sousa-Santos *et al.*, 2006b), even for females of other drainages.

In trials, each type of stimulus individuals was isolated and randomly positioned in each arm of the test aquarium, separated by acrylic plates. The females in test were positioned in the central zone of the aquarium, i.e. in the neutral one, and could freely swim into all choice compartments. To distinguish the type of stimulus used in mate recognition, different types of acrylic plates were used to isolate stimulus individuals, being carried out three different sets of trials. In the first one (from 30 April to 7 May 2009 and from 28 May to 1 June 2009), stimulus individuals were separated from the females in test by a perforated transparent acrylic plate (holes 4 cm apart from each other and with 5 mm diameter), allowing the passage of all types of cues between individuals. In the second set of trials (19-27 May 2009), the passage of visual cues was prevented by the use of two parallel perforated opaque black plates (the front one with the same grid of holes presented before and the holes from the second one, 2 cm apart from each other and with the same diameter, being not coincident with the ones from the front acrylic plate, thus totally preventing the passage of visual cues, but allowing the passage of chemical and acoustical ones). In the third set of trials (10-19 June 2009), stimulus individuals were isolated by transparent acrylic plates, not perforated and totally sealed, preventing the passage of water between compartments, thus only allowing the visual communication between individuals. Note that, although the use of acoustical cues was not specifically studied in present work, the trial sets in which perforated acrylic plates were used, theoretically, allowed the passage of such stimuli (if existing), thus the trials of the second set (visual stimuli passage prevented) will be, from now on, denominated as “chemical and acoustical trials”.

The same group of eleven females (from Mondego) were tested in the three described sets of trials, though each of these females was tested only once in each trial. However, due to sampling issues, some of the stimulus individuals were used more than once in the same set of trials. Therefore, a total of 33 mate choice trials were recorded, being each female tested three times.

After recording the three sets of mate choice trials, the same females were submitted to control tests, using the three different types of acrylic plates described before, but no stimulus individuals were present in the respective compartments. These tests allowed the assessment of possible preferred locations inside the test aquarium by females, unrelated to stimulus individuals. Therefore, a total of 33 control tests were again recorded, being each female tested three times.

All tests described above had one hour duration with 30 minutes prior habituation period to the test aquarium. Therefore, 66 hours of trials were recorded in the present work, being each female tested 6 times.

After each mate choice test, all individuals used were measured and weighted and later returned to the respective maintenance aquaria. The water of the test aquarium was, then, removed and the referred aquarium cleaned, dried and once again filled with new water, thus removing any kind of traces left by the previously used individuals. To ensure the health of the fish in test, the new water placed on test aquarium was always treated with Haloex from Waterlife trademark, following the manufacturer instructions. Note that, during tests, no filtering or aeration systems were used.

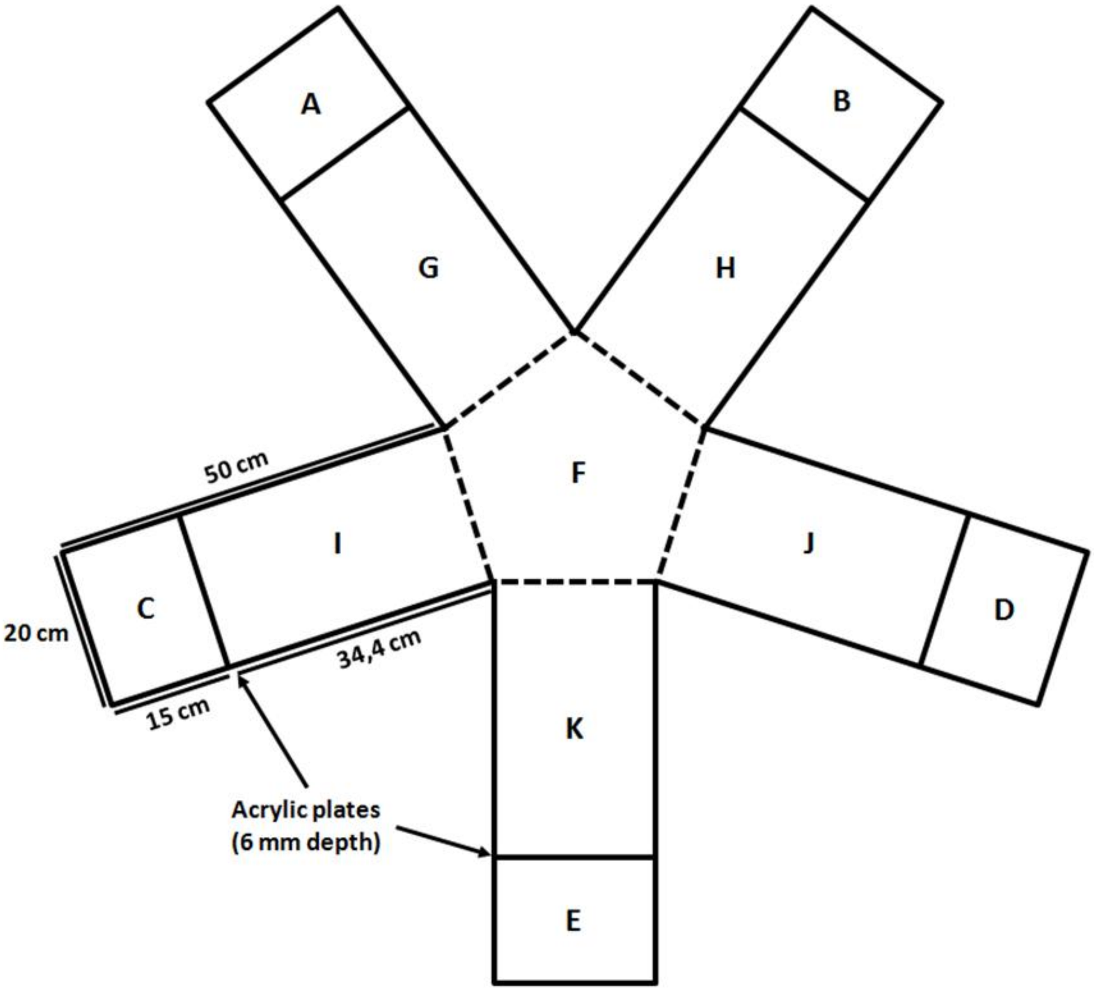

Figure 1. Test aquarium specially designed for this study, adapted from Schlupp *et al.* (1994). Legend: A – E: Stimulus individuals' compartments; F: Neutral compartment; G – K: Choice compartments. Neutral and choice compartments have all the same water volume.

Recordings analysis

The obtained videos were treated using JWatcher software (version 1.0) (Blumstein *et al.*, 2000). This software allowed the quantification of the time spent by each female in each of the six compartments of the test aquarium, namely, the five choice compartments and the neutral zone (central region of the test aquarium). Continuous sampling was used in all trials analysed. Tested females were only considered as entered a specific compartment when totally inside it, i.e. it was only considered as a compartment changing when the caudal fin of the female in test passed the lines that separate the choice compartments from the neutral one. Three quantifying measures were used in the analysis, namely, a) average permanence duration; b) maximum permanence duration; and c) permanence proportion.

Statistical procedures

All statistical procedures described below were performed with STATISTICA (version 8.0) software (StatSoft, 2008). Primarily, the three quantifying measures were tested for normality using a Shapiro-Wilk's W test. The non-normal variables were transformed to approach a normal distribution. In the normalized variables, the significance of the differences obtained between samples was assessed using repeated measures ANOVA. The variances' homogeneity between samples was analysed using a Levene's test. When a significant difference was found (α=0.05), a multiple comparison using Tukey HSD test was applied to assess which samples were responsible for the observed differences. The non-normal variables were compared using a Friedman ANOVA. Relations between female preference and stimulus individuals' body length and weight were analysed using Spearman's rank correlation coefficient (ρ).

Results

In what concerns fish gender determination, all symmetric tetraploids and non-hybrids (CCAA and AA, respectively, N=10 and N=7) matured before the behavioral tests, so all specimens used were confirmed as males. In what concerns *S. carolitertii* individuals (CC), none of them (N=3) matured during experiments, so their gender was further determined through gonads microscopic observation, which revealed that they were females. Regarding all triploid individuals (CAA and CCA, respectively, N=11 and N=2), only two CAA matured and were confirmed as females. However, the gender of all other triploids was predicted based on population data from previous studies performed in the same sampling locations (Pala & Coelho, 2005; Cunha *et al.*, 2008). Thus, the non-matured CAA individuals (*N*=9) were all considered females, since Pala and Coelho (2005) observed that, in River Alva (the Mondego sampling location), 93.6% of the triploids corresponded to females (only 10 males were found in a sample of 157 triploids). Besides, gonads' microscopic observation of seven triploid individuals not used in the behavioral tests, but from the same sample, revealed that all of them were females. In turn, CCA individuals were both considered males, since Cunha *et al.* (2008) observed that all captured CCA triploids in River Paiva were males. Note that gonads' smears of all triploid individuals used in behavioral tests could not be performed since their specific reproductive mode(s) will be further assessed in next year reproductive season.

The various stimulus groups showed differences in body length and weight with AA and CCAA individuals being the smallest and lighter ones, and CAA, CCA and CC individuals the bigger and heavier (Figure 2). As expected, body length and weight were highly correlated (ρ=0.93, *N*=33, *p*<0.05).

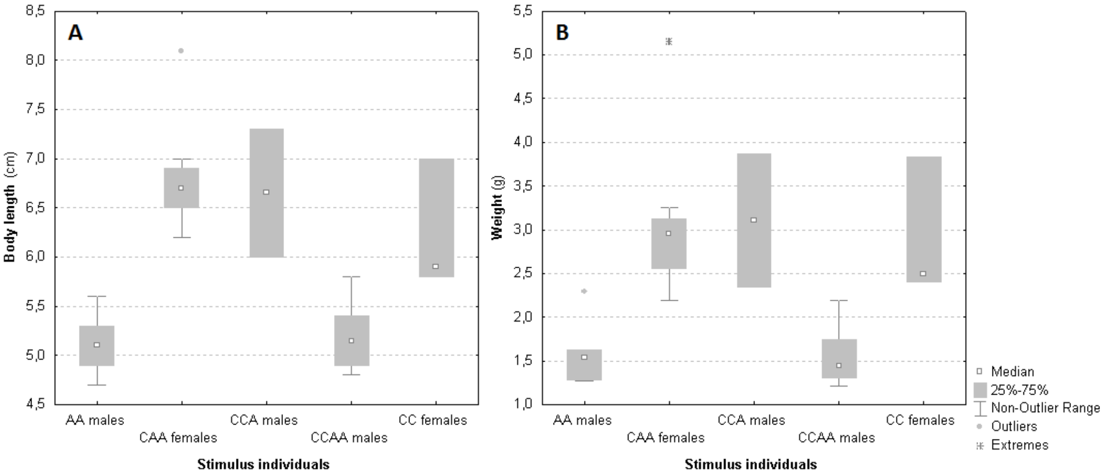

Figure 2. Body length (A) and weight (B) of the stimulus individuals used, namely, AA (males, *N*=7), CAA (females, *N*=11), CCA (males, *N*=2), CCAA (males, *N*=10) and CC (females, *N*=3).

Data on average and maximum permanence duration and permanence proportion per each type of stimulus individuals or choice compartment for all sets of trials are presented in Table III.

Multichannel communication trials

The analyses of the data obtained from the control trials, recorded with no stimulus individuals present in the test aquarium, showed no significant differences between choice compartments for all quantifying measures analysed, namely, average permanence duration (F=0.36, df=4, p>0.80), maximum permanence duration (F=0.30, df=4, p>0.85) and permanence proportion (χ^2=0.22, df=4, p>0.95). The data from average and maximum permanence duration were previously normalized using, respectively, 0.175 and 0.25 power transformations. The results from Levene's tests showed variances' homogeneity for average (F=0.53, df=4, p>0.70) and maximum (F=0.19, df=4, p>0.90) permanence durations.

In what concerns test trials with stimulus individuals present in choice compartments, no significant difference was found in average permanence duration between stimulus individuals types (χ^2=2.80, df=4, p>0.55). However, significant differences were detected in maximum permanence duration (F=2.76, df=4, p<0.045) and permanence proportion (F=2.65, df=4, p<0.05). The data from the last two quantifying measures were previously normalized using, respectively, a 0.3 power and an arcsine square-root transformations. The results from Levene's tests showed variances' homogeneity for maximum permanence duration (F=1.32, df=4, p>0.25) and for permanence proportion (F=2.49, df=4, p>0.05). The multiple comparison using Tukey HSD test revealed a near significant difference between CC and AA (df=40, p<0.055) and between CC and CCAA (df=40, p<0.065) permanence proportions. In what concerns maximum permanence duration, Tukey HSD test revealed a significant difference between CC and AA groups (df=40, p<0.05) and a near significant difference between CC and CCAA groups (df=40, p<0.055).

A more comprehensive analysis of permanence proportion data revealed that CAA and CCA samples showed an outlier each on the right side of their distributions, which were removed to perform a new statistical analysis. No transformation was needed to normalize this new variable, since it had a normal distribution. The outliers removal increased the significance of the differences (F=4.99, df=4, p<0.004). The results from the multiple comparison using Tukey HSD test revealed that CC sample differed significantly from all others samples (p<0.03 for all comparisons). However, according to Levene's test, the variances between samples were heterogeneous (F=6.33, df=4, p<0.001). The arcsine square-root transformation did not help the compliance of this assumption. The non-parametric Friedman ANOVA showed a near significant difference between samples (χ^2=9.10, df=4, p<0.06).

A significant correlation (p<0.05) between permanence proportion and stimulus individuals' body length (ρ=0.31, N=55) and weight (ρ=0.31, N=55) was found, though average and maximum permanence duration were not significantly correlated with those variables.

Chemical and acoustical communication trials

The analyses of the data obtained from the control trials, recorded with no stimulus individuals present in the test aquarium, showed no significant differences between choice compartments for all quantifying measures analysed, namely, average permanence duration (χ^2=5.35, df=4, p>0.25), maximum permanence duration (F=1.11, df=4, p>0.35) and permanence proportion (χ^2=4.37, df=4, p>0.35). The data from maximum permanence duration were previously normalized using a 0.25 power transformation and results from Levene's tests showed variances' homogeneity (F=0.51, df=4, p>0.70).

Regarding the test trials with stimulus individuals present in the choice compartments, no significant difference was found between stimulus individuals types in any of the three quantifying measures analysed, namely, average permanence duration (χ^2=6.90, df=4, p>0.10), maximum permanence duration (F=2.15, df=4, p>0.09) and permanence proportion (χ^2=3.77, df=4, p>0.40). The data from maximum permanence duration were previously normalized using a 0.25 power transformation and the results from Levene's tests showed variances' homogeneity (F=0.08, df=4, p>0.95).

No significant correlations were found between any quantifying measures and stimulus individuals' body length and weight.

Visual communication trials

The analyses of the data obtained from the control trials, recorded with no stimulus individuals present in the test aquarium, showed no significant differences between choice compartments for all quantifying measures analysed, namely, average permanence duration (χ^2=5.26, df=4, p>0.25), maximum permanence duration (F=0.92, df=4, p>0.45) and permanence proportion (χ^2=4.56, df=4, p>0.30). The data from maximum permanence duration were previously normalized using a 0.3 power transformation and the results from Levene's tests showed variances' homogeneity (F=0.62, df=4, p>0.60).

In what concerns test trials with stimulus individuals present in the choice compartments, no significant difference was found between stimulus individuals types in any of the three quantifying measures analysed, namely, average permanence duration ($\chi^2=2.50$, $df=4$, $p>0.60$), maximum permanence duration ($F=0.39$, $df=4$, $p>0.80$) and permanence proportion ($\chi^2=1.14$, $df=4$, $p>0.85$). The data from maximum permanence duration were previously normalized using a 0.3 power transformation and the results from Levene's tests showed variances' homogeneity ($F=1.15$, $df=4$, $p>0.30$).

No significant correlations were found between any quantifying measures and stimulus individuals' body length and weight.

Table III. Mean values of the three quantified variables (average permanence duration, maximum permanence duration and permanence proportion) for all trial sets.

Trial set	Test type	Choice compartments or Stimulus individuals	Quantifying measures		
			Average permanence duration (min)	Maximum permanence duration (min)	Permanence proportion
Multichannel	Control	1	3.56	8.96	0.2358
		2	2.67	8.43	0.2333
		3	1.06	4.75	0.1988
		4	1.11	4.55	0.1683
		5	0.79	3.11	0.1426
	Choice	AA (♂)	0.73	2.07	0.0937
		CAA (♀)	1.24	5.10	0.1718
		CCA (♂)	1.27	5.70	0.2207
		CCAA (♂)	0.76	1.70	0.0913
		CC (♀)	12.04	15.97	0.3776
Chemical and acoustic	Control	1	0.63	3.19	0.1583
		2	1.53	7.06	0.2943
		3	0.49	1.95	0.1051
		4	1.22	4.98	0.1995
		5	2.21	6.91	0.2092
	Choice	AA (♂)	6.73	11.43	0.2808
		CAA (♀)	0.60	3.22	0.1483
		CCA (♂)	1.94	7.67	0.2160
		CCAA (♂)	0.39	2.46	0.1106
		CC (♀)	1.67	4.61	0.1768
Visual	Control	1	1.16	4.80	0.2435
		2	0.80	3.34	0.1877
		3	6.68	11.14	0.2661
		4	0.63	2.74	0.1260
		5	1.18	3.95	0.1614
	Choice	AA (♂)	0.27	1.25	0.0934
		CAA (♀)	0.81	4.79	0.2627
		CCA (♂)	5.69	7.21	0.2020
		CCAA (♂)	0.67	4.92	0.1715
		CC (♀)	5.89	8.17	0.2210

Discussion

The three sets of control trials showed the inexistence of preferred choice compartments only related to spatial position. Thus, the patterns found in choice trials can be interpreted as a preference for the stimulus individuals present in the respective compartments. Note that, in most control trials, tested females did not equally divide the test time through the various compartments in the aquarium, staying more time in some compartments than in others. However, all compartments showed, in average, the same permanence time in all three types of communication trials (chemical and acoustical, visual and multichannel).

The analyses of the mate choice tests allowing the passage of multiple stimuli (visual, chemical and acoustical) revealed that, for two distinct quantifying measures (maximum permanence duration and permanence proportion), CAA females showed a preference towards individuals of the sympatric *Squalius* species from northern drainages (the distribution area of the tested females), i.e. *S. carolitertii*. However, the several isolated stimuli types seemed to be insufficient to identify preferences, once in chemical and acoustical and in visual trials the same tested females did not show a significant preference for any of the several available stimulus individuals. Thus, as observed in other species, not only several types of stimuli seem to be involved in communication between individuals during intersexual selection, but also the interaction between stimuli seems to play a fundamental role (reviewed in Rowe, 1999). In all limited communication tests performed, stimuli passage limitation was mutual between stimulus individuals and tested females, i.e. from stimulus individuals to females in test, but also from these ones to stimulus individuals. Considering that the main goal of the distinct trial sets was to determine which stimuli were involved in mate selection by females, ideally, stimuli passage should only be limited from stimulus individuals to tested females, since the occurrence of male complete courtships could be dependent on the reception of multiple stimuli issued by females. However, the conception of such experimental design is extremely difficult.

The significant differences found between the several types of stimulus individuals were due to the discrepancy between CC and AA and between CC and CCAA individuals, since tested females' permanence in CC's choice compartments was not significantly different from their permanence in those of CCA males and CAA females. However, for permanence proportion variable, after removing the only two detected outliers in all samples (present in CCA and CAA groups), the permanence of tested females in CC individuals' choice compartments became significantly higher than in all other stimulus individuals (Figure 3). Thus, it was clear that CAA females showed a preference for *S. carolitertii* over all other individuals used as stimulus. For CAA stimulus females (the same ones that were tested), the only high permanence proportion value found might be due to the higher familiarity existing between these females, since they were kept together in the same division of the maintenance aquarium. However, this behavior was only found in one tested female. Non-hybrid (AA) and symmetric tetraploid (CCAA) males were the less preferred by CAA females for all quantifying measures analysed (Table III). The non-preference for AA males was somehow expected, since this male type is absent in northern populations (reviewed in Alves *et al.*, 2001; Pala & Coelho, 2005; Cunha *et al.*, 2008). Some works in fish species have shown that females can distinguish between males from their own populations and foreign ones (Ptacek & Travis, 1997; Wong *et al.*, 2004), which may be the case of *S. alburnoides* complex, since AA males are phenotypically distinct (Collares-Pereira, 1984). Anyway, the more vigorous body quivering and faster maneuvers possibly made by these males towards females due to their smaller size do not seem to explain their apparently higher reproductive success in southern populations (Sousa-Santos *et al.*, 2006b), which is probably related to the exhibition of sneaking behaviors.

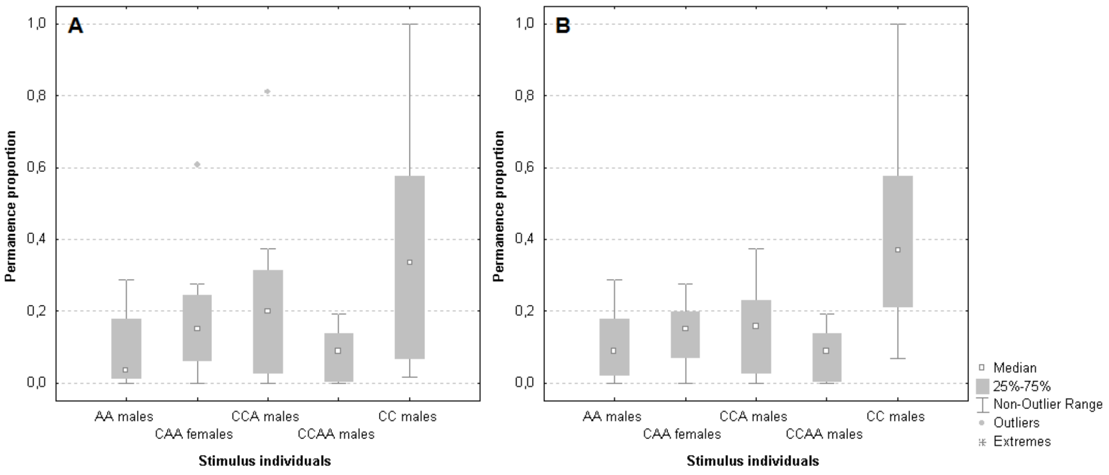

Figure 3. Permanence proportion of tested females in each of the choice compartments of the respective stimulus individuals, respectively, with all data (A) and after outliers' removal (B) (see text for explanation).

The posterior observation of the immature gonads revealed that the three *S. carolitertii* used were females. Thus, at first glance, the clear preference of tested females for individuals of this sympatric species seems of difficult comprehension.

However, the obtained results leave no room for doubt about such preference pattern (Figure 3), certainly not being a random effect. In one hand, it could be postulated that this preference would be only due to a female grouping tendency, not revealing any evidence about reproduction choice. Actually, in some fish species, females show an aggregation tendency to avoid male harassment and, simultaneously, to avoid predators through numerical dilution and/or increased vigilance (Pilastro *et al.*, 2003; Dadda *et al.*, 2005; Agrillo *et al.*, 2006; Plath & Tobler, 2007). However, *S. carolitertii* females were not the only present as stimulus individuals, thus tested females could have grouped with triploid *S. alburnoides* stimulus females (with which they shared the same genomic constitution, CAA). Since such grouping was not observed, the preference pattern revealed in this work might have other biological explanations, besides the simple female aggregation, namely a reproduction tendency with *S. carolitertii* males. If there are clear advantages for CAA females in reproducing with these males (which will be discussed below), there are several possible explanations for their association with *S. carolitertii* individuals, regardless of their gender. In bisexual species, sexual recognition is mediated by different stimuli types (reviewed in Myrberg, 1980; Plath *et al.*, 2003; Turnell *et al.*, 2003; Thompson *et al.*, 2004; Gammon *et al.*, 2005). Therefore, the stimuli that allow *S. carolitertii* individuals to intraspecifically discriminate their sex may not be recognizable by *S. alburnoides* females. Therefore, considering the several benefits of their reproduction with males of the bisexual species, *S. alburnoides* females may take advantage on the indiscriminate search of *S. carolitertii* individuals, regardless of their gender. The incapacity of CAA females on sexually discriminate *S. carolitertii* individuals may be because they are not from the same “pure” species. In one hand, not enough time may have elapsed to evolve interspecific sexual recognition mechanisms. On the other hand, perhaps the disadvantages of chasing *S. carolitertii* individuals regardless of their sex may not be significant enough for natural selection to favor the appearance of such sexual recognition mechanisms. This hypothesis is corroborated by the fact that, when existing, agonistic aggressive behaviors usually occur between males, thus the accidental encounters between females may not lead to high costs for them. Moreover, sexual recognition sometimes occurs only through direct contact between individuals (Myrberg, 1980; Plath *et al.*, 2003) and this would explain the non--recognition between genders in the present case, since the experimental design did not allowed the direct contact between females in test and stimulus individuals. Thus, *S. alburnoides* females seem to recognize *S. carolitertii* phenotype, but may be incapable of discriminating genders without a close contact. Another possible explanation for the lack of sexual recognition capacity by CAA females may lie on the fact that *S. carolitertii* individuals were not mature during tests, since, in most species, sex recognition seems to be only possible when individuals are sexually receptive (reviewed in Myrberg, 1980). Finally, it was demonstrated for a marine fish that inexperienced females follow the most experienced to learn the routes to mating sites (Warner, 1990). If the same strategy is adopted, it is possible that *S. alburnoides* females take advantage on aggregating with *S. carolitertii* females, since they certainly know better the species mating locations and optimal habitats, thus increasing their probability of finding *S. carolitertii* males, what may have several adaptive advantages as discussed below.

Therefore, given all explanatory hypotheses described above, the obtained results will be from now on discussed considering that the association between tested females and *S. carolitertii* females does reveal a tendency for a mating preference of CAA females for *S. carolitertii* males.

To choose a particular mate, females must recognize particular phenotypic traits that allow the discrimination between individuals. As referred, such phenotypic traits are directly related to adaptive differences. According to present results, it seems that male dimensions and spatial segregation play a key role in sexual selection.

A significant positive correlation was detected between permanence proportion and stimulus individuals' weight and body length. Thus, CAA females seem to prefer bigger males. Although male body size has more influence on intrasexual selection in many groups (Andersson, 1994), female preference for bigger males was already reported in other species (Cote & Hunte, 1989; Zimmerer & Kallman, 1989; Ptacek & Travis, 1997; Reichard *et al.*, 2005). A mate preference linked to male dimensions in *S. alburnoides* has a special importance because some forms differ in body size, which can lead to differences in males' reproductive success. More specifically, the positive correlation found between female preference and male body size may be due to their preference for *S. carolitertii* males, which attain bigger dimensions than *S. alburnoides* (Cunha *et al.*, 2009), although the CC stimulus individuals used in this study were not significantly bigger than CAA stimulus females or CCA stimulus males (Figure 2). Therefore, the adaptive advantages inherent on reproducing with *S. carolitertii* males may have led to the evolution of a preference for bigger males, facilitating CC males' selection. Moreover, such preference may justify the two observed outliers present in CAA and CCA stimulus individuals' samples for permanence proportion, since these groups are the biggest within the complex. Thus, tested females may have made a wrong choice based, mainly, in body size. Furthermore, this preference for bigger males also explains the non-preference for non-hybrid and symmetric tetraploid males, which were significantly smaller than all other forms (Figure 2). However, as already referred, the isolated visual stimuli were not enough to define a preference for any type of stimulus individuals, thus, although relevant, body size alone does not seem to be sufficient to choose a mate.

Besides the apparent relation between female preference and male dimensions, spatial segregation between forms seems also to play a key role in sexual selection, since, during *S. alburnoides* reproductive period (Ribeiro *et al.*, 2003), triploid females and *S. carolitertii* individuals concentrate preferentially in habitats with the same characteristics, namely, higher current velocity and instream cover proportion, showing the other *S. alburnoides* forms distinct optimal habitats (Martins *et al.*, 1998; Santos *et al.*, 2004). Thus, during reproductive period, triploid females seem to search for locations where there is a higher probability of finding their preferred males. On the other hand, preference for *S. carolitertii* males might have arisen from their higher availability in CAA females' optimal habitats, thus this strategy is likely to have adaptive advantages being consequently under positive selection.

Indeed, the preference for *S. carolitertii* males by *S. alburnoides* CAA females may well have direct consequences for the genetic and population dynamics of this hybridogenetic complex.

In what concerns genetic dynamics, the occurrence of such crosses explains the total substitution in *S. alburnoides* specimens from northern drainages of the typical *S. pyrenaicus* maternal genome by the *S. carolitertii* one without the occurrence of the same levels of mitochondrial DNA introgression from that sympatric species. Thus, the nuclear genes introgression from the various sympatric *Squalius* species into the complex seems to have resulted from interspecific crosses involving males from those species and *S. alburnoides* females, which present, in general, *S. pyrenaicus* mitochondrial DNA. Note that, even if other *S. alburnoides* female forms are likely to have distinct mate choices, the male preference of the CAA ones can be of extreme importance for the complex population dynamics, since they are the most common form in natural populations, certainly playing a key role in populations' structure.

Furthermore, from the evolutionary point of view, there are inherent genetic advantages on reproducing with individuals of a bisexual species, since their gametogenesis occurs through normal meiosis, with recombination and, consequently, an increase of genetic variability. Thus, the heterospecific reproduction between *S. alburnoides* and *S. carolitertii* ensures the entrance of new genetic variation into the complex, raising its adaptive capacity and, consequently, its success through time. Actually, in males from northern populations, meiotic recombination only occurs in the gamete production of symmetric tetraploids, rare in most populations, since all other *S. alburnoides* males have a non-sexual reproductive mode, namely, clonal gamete production (Pala & Coelho, 2005; Sousa-Santos *et al.*, 2007b). However, though symmetric tetraploid males produce their gametes through normal meiosis, the present work demonstrated that CAA females do not show any clear tendency in reproducing with them. The explanation for this may lay on the fact that symmetric tetraploids could be produced without meiotic recombination, once, in most crosses that originate this offspring type, none or just one of the progenitors produced its gametes through normal meiosis or meiotic hybridogenesis, reproductive modes that ensure recombination.

Besides the evident relation between the referred interspecific crosses and *S. alburnoides* genetic dynamics described above, its population dynamics seems also to be highly affected by intersexual selection, namely as regards population composition. Since the most frequent female type shows a preference for a particular male form, the majority of its offspring will show the same specific genomic constitution. Thus, it is easily conceivable that the composition and evolution of *S. alburnoides* populations will be strongly conditioned by females' mate preferences, namely, by the most common ones.

To explain the actual composition of *S. alburnoides* natural populations based on the observed reproduction tendencies, it is strictly necessary to know the reproductive mode(s) of the involved forms to predict the genomic constitution of the offspring. Triploid females' gamete production has been largely studied in southern populations (PAA genome), being demonstrated that these females show distinct reproductive modes. More specifically, it was evidenced the simultaneous production of haploid (A genome) and triploid (PAA genome) oocytes by the same triploid female, respectively, produced by meiotic hybridogenesis and clonally (Alves *et al.*, 2004). Other studies also demonstrated the production of diploid oocytes (AA genome) by PAA females (reviewed in Alves *et al.*, 2001), thus proving the existence of three distinct reproductive modes in triploid females, although meiotic hybridogenesis seems to be the most common. However, very little is known about gamete production of northern triploid females (CAA genome). Pala & Coelho (2005) demonstrated that these females can produce their gametes through meiotic hybridogenesis. However, only one CAA female was tested, thus the findings of the referred work cannot be generalized. Therefore, as happens with the triploid ones from the South, CAA females may present alternative reproductive modes, which directly influence the interpretations at population level of the mating preferences now found.

If CAA females' oocytes are produced through meiotic hybridogenesis, their offspring will be mostly constituted by diploid hybrids (CA), since their preferred *S. carolitertii* males (CC) produce, through normal meiosis, haploid sperm with C genome. Actually, in northern populations, all diploid hybrids are likely to descend from the referred crosses, i.e. involving CAA females and *S. carolitertii* males, since no other crosses between any of the available forms in these populations produce CA offspring. The production of diploid hybrids involves, in general, non-hybrid males as progenitors, which occurrence is highly unlikely because they only exist in southern populations.

However, the frequency of diploid hybrids in northern populations is lower than expected (Table I), considering the results obtained in this study, i.e. the theoretical preference for *S. carolitertii* males by CAA females. At first glance, the possible differential viability and/or survivorship between offsprings could somehow justify the low proportion of diploid hybrids. However, from the evolutionary point of view, this hypothesis seems unlikely, since, being true, the preference for *S. carolitertii* males by CAA females would not have been selected through time.

Besides the lower than expected frequency of diploid hybrids, the proportion of CAA females in most northern populations is higher than predicted, considering the results of the present study and the existence of meiotic hybridogenesis as a main reproductive mode. CAA form maintenance in northern populations is restricted to crosses involving females with the same genomic constitution and males that produce CA sperm, i.e. diploid hybrid (CA) or symmetric tetraploid (CCAA) males (whose gametes are formed, respectively, through clonal gametogenesis and normal meiosis). Therefore, according to CAA females' preference for *S. carolitertii* males and to the current low frequency of diploid hybrid and symmetric tetraploid males in most populations, the predominance of CAA females might not prevail through time. Note that CAA females' preference for diploid hybrid males was not tested in the present work (because no CA individuals were found in the collected samples as a consequence of being uncommon in most populations) and, consequently, it is not possible to discard that triploid females would prefer the diploid hybrid males (if available). However, being quite rare, it is unlikely that diploid hybrids might play an important role in *S. alburnoides* current population dynamics.

Therefore, considering the unexpected deviations in populations' composition described above, it seems likely that *S. alburnoides* populations with CAA females' predominance are not yet stable and should evolve in reducing their frequency and increasing the proportion of diploid hybrids. In turn, it is possible that a population with an increase in the frequency of diploid hybrids should soon increase the proportion of symmetric tetraploids, once, in most cases, their offspring present such genomic constitution (Table II).Thus, if sexual selection occurring in symmetric tetraploids leads to their auto-selection, *S. alburnoides* populations may present a strong tendency to tetraploidization, as supported in other studies (Alves *et al.*, 1999, 2004; Cunha *et al.*, 2008). However, this approach is highly speculative and can only be verified through the study of diploid hybrids and symmetric tetraploids sexual selection.

All the discrepancies found between current populations' composition and the theoretical predictions described above may suggest that CAA females do not produce their gametes through meiotic hybridogenesis. Thus, if CAA females produce their oocytes through simple hybridogenesis (Table II), crosses involving the preferred *S. carolitertii* males would lead to the production of CAA offspring, justifying the high predominance of this form in northern populations. On the other hand, if gamete production is clonal in CAA females, their offspring will only be viable (ploidy ≤ 4n) in crosses with males that produce haploid gametes. However, none of the *S. alburnoides* male types present in northern populations produce haploid sperm (Table II), so the only viable offspring produced by CAA females would involve crosses with *S. carolitertii* males, justifying their preference for such males. Thus, all offspring produced by CAA females would correspond to symmetric tetraploids, which corroborates *S. alburnoides* tendency for tetraploidization, even more marked if symmetric tetraploids would select themselves as sexual partners, producing the same form as offspring.

Considering all possible population patterns described above, simple hybridogenesis seems likely to be the most common reproductive mode in CAA females, once it is the most consistent with the northern populations' composition and with the results obtained in the present study. Therefore, assessing, in a quantitative way, the reproductive mode(s) of CAA females is of extreme importance at this stage, thus offspring production of the tested females that were kept alive will be studied in the next year reproductive season.

The population patterns that were discussed confirm the relevance of studying reproductive behavior for the comprehension of *S. alburnoides* dynamics and evolutionary success, but further behavioral testing is essential to clarify some aspects that remain unclear, namely: i) the study of auto-sexual selection in diploid hybrids and symmetric tetraploids that can bring some important clues for the assessment of *S. alburnoides* tetraploidization tendency; ii) the possibility that CAA females prefer diploid hybrid males instead of *S. carolitertii* ones; and iii) the intrasexual selection that may also play a key role in population dynamics since there are marked phenotypic differences between the various male types. Moreover, analysing the viability and survivorship of offsprings produced by the different types of crosses is another step in behavior-related studies because they may allow confirming some of the presented assumptions.

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Concluding Remarks

The results of this thesis allowed demonstrating the important role of intra and interspecific reproductive behavior in *S. alburnoides* dynamics.

Briefly, a non-intrusive individual recognition system based on scale patterns was proposed. In what concerns reproductive behavior, the study revealed that the most common *S. alburnoides* female form seems to have a preference for males of the sympatric *S. carolitertii*, which brought some relevant clues for the comprehension of complex's genetic and population dynamics.

Moreover, the obtained results raised new questions, not only related with the complex's reproductive behavior, but also with other associated topics, namely: Do triploid females prefer *S. carolitertii* males over the diploid hybrid ones? What are the mate preferences of other female forms? Do diploid hybrids auto-select themselves as sexual partners? And do symmetric tetraploids act identically? How does intrasexual selection interfere with intersexual selection? Do distinct offsprings have differential viability/survivorship? Do northern triploid females present alternative reproductive modes as observed in southern populations? Answering to these questions might play a key role in the follow up process of clarification of *S. alburnoides* dynamics.

Furthermore, other issues were poorly understood because of some experimental design limitations. For example, nothing could be inferred regarding the use of acoustical stimuli in the communication of individuals during intersexual selection, which may be tested through the assessment of the emission of such waves' type by *S. alburnoides* forms. Besides, some issues are still open concerning the existence of interspecific sexual recognition of *S. carolitertii* individuals by triploid females, requiring some further studies. Although the obtained results suggest that such process does not occur, this cannot be guaranteed, since the absence of sexual recognition observed might be due to the lack of direct contact between the respective individuals and/or to the fact that such individuals were not mature during behavioral tests.

Notwithstanding, the present research on the complex reproductive behavior may be also considered of great significance as regards the conservation of *S. alburnoides*, since it was listed as "Vulnerable" in the National Red Data Book (Cabral *et al.*, 2005). Indeed, their specific evolutionary success is likely dependent on the sympatric *Squalius* species, namely, *S. pyrenaicus* and *S. aradensis* in the south, which are also of high conservation concern, and *S. carolitertii* in the north. Besides, being a well succeeded hybridogenetic complex, *S. alburnoides* is considered of high scientific interest, which adds value to its conservation. Moreover, identifying the reproductive strategies of the main forms that occur in natural populations and their spatial and temporal contexts is of high relevance to define accurate action plans aiming the maintenance of both northern and southern populations. The comprehension of such reproductive strategies is of extreme relevance for conservation efforts, once the maintenance of *S. alburnoides* forms is closely interdependent, thus the decrease of one form's relative frequency may imperil the formation of others and, consequently, endanger the population future. Thus, conservation priorities should be established and framed on the accurate knowledge on populations' dynamic, both at intra and interspecific levels, if the objective is to preserve the extant biodiversity and the ongoing evolutionary processes.

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