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Fertile Triploid Males—An Uncommon Case Among Hybrid Vertebrates

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ABSTRACT The endemic Iberian minnow *Squalius alburnoides* is a complex of fishes of hybrid origin including both males and females with distinct ploidy levels and varying proportions of the parental genomes. In this paper we demonstrated that in contrast to many vertebrate hybrid lineages the sperm of triploid hybrid males of *S. alburnoides* is viable and fully functional. Flow cytometry and analysis of sequences of a fragment of the beta-actin nuclear gene applied to progenitors and offspring evidenced that these males produced their sperm clonally, as already described for diploid hybrids. The presence of different types of fertile males (nonhybrid diploids with normal meiosis and both diploid and triploid hybrids) coupled with hybridogenetic meiosis in females endows this vertebrate complex with a high level of independence from other species and contributes to maintain its genetic variability. *J. Exp. Zool. 307A:220–225, 2007.* © 2007 Wiley-Liss, Inc.

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Often when two distinct species hybridize, there is a disruption of normal meiosis that leads to partial or total infertility of the descendants (Vrijenhoek, '89, '94). Some hybrids, however, overcome this problem by reproducing by diverse gametogenetic mechanisms. These alternative modes of reproduction among hybrid lineages of vertebrates are known to have evolved independently in many groups and may involve asexual or modified forms of sexual reproduction (reviewed in Dawley and Bogart, '89).

Within the fish family Cyprinidae, the small Iberian minnow *Squalius alburnoides* is an example of an ecologically extremely well-succeeded hybrid lineage that was originated by intergeneric crosses between *Squalius pyrenaicus* females—P genome—and males of a probably extinct paternal species—A genome (Alves et al., 2001), phylogenetically close to the extant *Anaecypris hispanica* (Robalo et al., 2006). This interesting female sex biased complex comprises diploid, triploid and tetraploid hybrid forms and a diploid nonhybrid form (AA) constituted almost exclusively by males (reviewed in Alves et al., 2001). This diploid AA male lineage contains two genomic complements inherited from the paternal ancestor but a *pyrenaicus*-like mtDNA (Alves et al., 2002), a clear indication that they resulted from the hybrids and are not ''survivors'' of the paternal progenitor of the complex. Instead of being a typical asexual all-female lineage that reproduces clonally, this complex includes also fertile males and has varied reproductive modes and gametogenetic processes.

Concerning the spermatogenetic processes, it is known that normal meiosis occurs in diploid nonhybrids (AA) and in balanced tetraploids (PPAA) and that on the contrary, diploid hybrids

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(PA) produce fertile unreduced sperm, transmitting the intact genome to the offspring (Alves et al., '98, '99). A study of two triploid males, which seem to be very rare in natural populations, by flow cytometry of the DNA content of erythrocytes and spermatozoa, showed that they also produced unreduced sperm (Alves et al., '99) but their genomic constitution (PAA or PPA) remained unknown and their sperm functionality was not tested.

Therefore, the aims of the present study were: (1) to clarify the reproductive mode and fertility of triploid males by accurately determining the genomic constitution of their sperm and of progeny using a suitable nuclear marker (beta-actin gene) combined with ploidy confirmation; and (2) to evaluate the viability of the progeny produced by triploid males. To validate the applied methodology, crosses with diploid PA and AA males were also performed.

MATERIALS AND METHODS

Five *S. alburnoides* males (one triploid, two hybrid diploids and two nonhybrid diploids) were selected after the determination of their genomic constitutions by beta-actin gene sequencing and the confirmation of their ploidy by flow cytometry. These males were then used in crossing experiments with *S. alburnoides* and *S. torgalensis* females and their live progeny ($N = 31$) was subsequently analysed, also by beta-actin gene sequencing and flow cytometry. Although DNA nuclear measurements were impracticable in very small dead juveniles, the DNA content of 33 additional preserved juveniles was analysed since ploidy assessment was also possible through the analysis of the beta-actin gene sequences (see below). The ploidy of the *S. torgalensis* females was not determined since it is expected to be a diploid species like *S. aradensis*, *S. carolitertii* and *S. pyrenaicus* (Collares-Pereira and Moreira da Costa, '99). This was confirmed by the fact that of more than 25 *S. torgalensis* sequenced for the beta-actin gene none gave indications of being nondiploid (*unpublished data*). Additional and more detailed methodological information is presented below.

Assessment of the genomic constitutions

When a nuclear gene of a hybrid between closely related species is sequenced, single nucleotide polymorphisms appear in chromatograms as single double peaks. However, in the case of a hybrid between species that differ by one or more indels in a particular gene fragment, the sequencing process generates artefacts in the resultant chromatograms that appear as series of false double peaks and it is possible to reconstruct the complements involved since the bases are out of phase for as many positions as the size of the indel—for more details see Sousa-Santos et al. (2005). This process of reconstruction of the genomes present in hybrids is favoured by a previous knowledge about some fixed mutations that are specific of the parental genomes as these act as reference points that make possible to ascribe unambiguously each peak to the correct parental genome (Sousa-Santos et al., 2005).

Thus, to evaluate the result of the spermatogenetic process of a *S. alburnoides* male through the observation of the genomic constitution of its progeny, it was considered ideal to cross the male with a female of a species having characteristic marker mutations absent in the male but sufficiently related to prevent reproductive isolation. This important role could be performed by *S. torgalensis* females from Mira River, where *S. alburnoides* complex is absent. Indeed, concerning the beta-actin gene of the Portuguese *Squalius* species, three groups of haplotypes were found so far, clearly distinct from each other by characteristic patterns of fixed mutations and indels (Sousa-Santos et al., 2005, 2006): P and C haplotypes in *S. pyrenaicus* and *S. carolitertii* (respectively); Q and T haplotypes in *S. aradensis* and *S. torgalensis* (respectively); and A haplotypes in *S. alburnoides*. At the beta-actin gene level, the P and C haplotypes are not distinguishable from each other and the same occurs between the Q and T haplotypes (Sousa-Santos et al., 2006). *S. alburnoides* hybrids exhibit a mixture of A and P, C or Q haplotypes depending on whether they are, respectively, descendants of *S. pyrenaicus*, *S. carolitertii* or of *S. aradensis*, the three *Squalius* species with which *S. alburnoides* occurs in sympatry.

Since all *S. alburnoides* males selected to be used in the crossing experiments were from Tagus and Douro river basins (where *S. pyrenaicus* and *S. carolitertii*, respectively, occur in sympatry with *S. alburnoides*), they presented similar beta-actin haplotypes (P and C). Crossings with *S. torgalensis* bearing beta-actin T-haplotypes that are clearly distinct from P and C haplotypes would, therefore, allow an easy discrimination of the genomes inherited by the progeny, a situation that would not occur if the females used in the experiments were *S. pyrenaicus* or *S. carolitertii* since they would bear P and C haplotypes like the *S. alburnoides* male progenitors.

The count method described by Sousa-Santos et al. (2005) was applied to a segment of 176 bp and the resultant *P*-count values were used to assess the ploidy and genome constitution of the *S. alburnoides* hybrids.

Experimental crosses

Each *S. alburnoides* male (one CAA triploid from the Douro River and two PA diploids from the Tagus River basin) was isolated with one *S. torgalensis* female (TT) from Mira River (crosses 1–3) in a tank (30 l) with vegetation and coarse substratum, under natural conditions of light and temperature, for 2 months (May and June 2005). The spawning was not artificially induced and the experimental pairs spawned naturally, after which they were removed from the tank to minimize the loss of eggs and juveniles by predation. The majority of the eggs was lost due to fungal infections but a total of 31 juveniles was reared to the age of 8 months to attain a sufficiently large body length (2–4 cm) that allowed their survival after blood and fin clip sampling. A total of 33 juveniles that died during this period were immediately preserved in 96% ethanol.

In addition to these controlled experiments, two juveniles (9-months-old) resultant from group spawnings between seven *S. alburnoides* AA males from Tagus River and eight *S. torgalensis* females from Mira River and eight juveniles (21-months-old) resultant from crosses between two AA males and two PA females of *S. alburnoides* from the Tagus river were also reared for comparative analyses (crosses 4 and 5). Due to their low number, only the beta-actin gene sequencing was performed since blood sampling of small fishes for flow cytometry procedures involves a high risk of mortality.

In total, considering the controlled experiments with only one pair of spawners and the experiments involving groups with more than one pair of spawners, 74 juveniles were sequenced which resulted from five crosses (two PAxTT, PAAxTT, AAxTT and AAxPA).

All the experiments were made in conformity to the ethical guidelines for animal care approved by the authors institutions.

Laboratory methods

Ploidy determination by flow cytometry was made using small blood samples drawn from the caudal vein of live juveniles or using fresh fin clips in the case of the adults used in the crossing experiments (since these measurements were made prior to the spawnings and the removal of fin clips involved less risks than blood sampling to the survival of the adults). The procedures used for blood samples were the ones described in Alves et al. ('99) and for the analysis of fin samples an adaptation of the method proposed by Lamatsch et al. (2000a) developed by M.J. Collares-Pereira et al. (*unpublished*) was used. The DNA content estimation for individual fishes followed the procedure described in Alves et al. ('99). Total genomic DNA was extracted from fin clips preserved in ethanol by an SDS/proteinase-k-based protocol, precipitated with isopropanol and washed with ethanol before re-suspension in water (adapted from Sambrook et al., '89). A total of 927 bp of the *beta-actin* gene was amplified using the primer BACTFOR and conditions described in Robalo et al. (2006). Sequences were aligned with BioEdit® (Ibis Therapeutics; Carlsbad, CA) v.5.0.6.

RESULTS

The analysis of the beta-actin gene sequences of the *S. alburnoides* males used in the crossing experiments that involved only one pair of spawners showed that they were all hybrids with both P and A complements. *P*-count values showed that males used in crosses 1 and 2 were diploids (PA genome) and that male of cross 3 was a triploid with a CAA genomic constitution. The flow cytometry analysis confirmed the ploidies of these three males (Table 1).

As shown in Table 1, all juveniles fathered by diploid hybrid males (crosses 1 and 2) were triploids with a TPA genomic constitution ($N = 43$). The descendants of the triploid male (cross 3) were all tetraploids with a TCAA genome ($N = 21$). This means that the sperm produced by the diploid and triploid males was also diploid and triploid, respectively. For the 31 offspring for which we had data from beta-actin sequencing and flow cytometry the results showed, without exception, full concordance between the two approaches.

Live progeny that have resulted from these three crosses reached 1 year of age and are still currently exhibiting an apparently normal development. These juveniles are being kept alive in order to try to evaluate their fertility and gametogenetic mechanisms.

Concerning the experimental crosses that involved more than one pair of spawners (crosses

TABLE 1. Genomic constitution and ploidy of the males used in the controlled crossing experiments and their respective progenies inferred by flow cytometry (DNA content of erythrocytes and/or fin cells) and beta-actin nuclear gene sequencing (P-counts) analyses

		Cross 1	Cross 2	Cross 3
S. alburnoides male	*P*-count	53.39%	61.02%	14.41%
	Erythrocytes (pg/cell)	2.68	2.73	3.85
	Inferred ploidy/genome	2*n*/PA	2*n*/PA	3*n*/CAA
Juveniles	*N* (live/preserved)	19 (2/17)	24 (16/8)	27 (13/8)
	P-count	89.70% ± 1.90 ($N = 19$)	94.55% ± 2.36 ($N = 24$)	63.83% ± 7.78 ($N = 21$)
	Erythrocytes (pg/cell)	3.80 ± 0.25	3.68 ± 0.12	5.23 ± 0.22
	Mean ± SD	($N = 2$)	($N = 16$)	($N = 13$)
	Inferred genome	TPA	TPA	TCAA

All crosses were mothered by *S. torgalensis* females (TT genome). P, C and A beta-actin haplotypes were inherited from the male progenitors. Flow cytometry reference values: 2.44 ± 0.08 pg/cell for diploids, 3.64 ± 0.16 pg/cell for triploids and 4.83 ± 0.08 for tetraploids (Alves et al., '99). *P*-count reference values: 55.77% ± 6.88 for diploids and balanced tetraploids and 95.69% for PPA triploids (Sousa-Santos et al., 2005).

4 and 5), both groups involved *S. alburnoides* males with an AA genome (mean 2.65 ± 0.10 pg/cell, $N = 7$; and mean 2.67 ± 0.21 pg/cell, $N = 2$, respectively). In cross 4 the nonhybrid males fertilized the eggs of *S. torgalensis* females and the resulting progeny was constituted by diploid TA hybrids (*P*-count = 64.41% ± 3.60, $N = 2$). In cross 5, the nonhybrid males mated with *S. alburnoides* females with a PA genome (mean 2.54 ± 0.01 pg/cell and *P*-count = 58.47% ± 1.20, $N = 2$) and the resulting progeny was triploid and exhibited a PAA genome (*P*-count = 5.93% ± 1.81, $N = 8$). This means that in both crosses, the AA males produced haploid sperm which fertilized, respectively, haploid eggs from the *S. torgalensis* females and diploid eggs from *S. alburnoides* PA females (as expected since meiosis does not occur in this type of females, as described by Alves et al., '98).

DISCUSSION

The occurrence of triploid vertebrates is considered to be a rare phenomenon and is generally coupled with infertility or, more rarely, with asexuality in allotriploids, since normal meiosis is likely to be disrupted by cytological mechanisms that preclude synapsis between heterospecific chromosomes (Dawley, '89; Stöck et al., 2002). Thus, the establishment and survival of these organisms depends on clonal reproductive modes such as parthenogenesis or gynogenesis; on the incorporation of unusual meiotic mechanisms (like hybridogenesis) that allow recombination to occur in triploid females, often with the exclusion of the genetic complement inherited from the male parent; and/or on the ploidy elevation by the incorporation of additional genomic material (processes detailed in Dawley, '89; Avise et al., '92; Vrijenhoek, '94; Beukeboom and Vrijenhoek, '98; Alves et al., 2001; Scali et al., 2003; Bi and Bogart, 2006).

Triploid males of several species often develop testes and successfully complete spermatogenesis but are in general sterile due to the production of aneuploid and/or abnormally shaped spermatozoa, although some may produce functional sperm and generate embryos with a very low survival rate. The sterility of triploid male fishes has been documented for both artificially induced triploids [e.g., *Rhodeus ocellatus ocellatus* (Kawamura et al., '99), *Carassius auratus* (Gui et al., '92), *Verasper moseri* (Mori et al., 2006), *Salvelinus fontinalis* (Allen and Stanley, '78) and *Oncorhynchus mykiss* (Thorgaard and Gall, '79)] and for naturally occurring individuals, whose triploidy resulted from the incorporation of additional genomic material by an unreduced egg [e.g., *Misgurnus anguillicaudatus* (Matsubara et al., '95; Zhang and Arai, '99; Oshima et al., 2005), *Poecilia formosa* (Lamatsch et al., 2000b) and *Tinca tinca* (Flajshans et al., '93)].

In contrast, the results presented in this study showed that a triploid CAA *S. alburnoides* male produced unreduced sperm (like the diploid PA males) which, after fertilization of haploid eggs from *S. torgalensis*, gave rise to viable tetraploid offspring. The fertilization by the unreduced sperm of these triploid males leads to ploidy elevation of the offspring, as described by Bogart et al. ('89) for hybrid salamanders of the genus *Ambystoma*. Interestingly, however, this process does not seem to increase indefinitely the maximum number of chromosomes of the complex since females seem to act as regulators of the ploidy level. Indeed, triploid *S. alburnoides* females, the most abundant form in natural populations, exhibit a mechanism of gamete production (meiotic hybridogenesis) that constitutes a limitation to an uncontrolled growth of the ploidy level: the ability to exclude the unmatched set of chromosomes is likely to enable, in general, a subsequent normal meiosis and the production of haploid eggs. The same type of reduction to a haploid stage was observed in fertile triploid males of the Batura toads all-triploid species *Bufo pseudoraddei baturae* (Stöck et al., 2002), in the water frog *Rana esculenta* triploid males (Graf and Pelaz, '89) and in *Rana nigromaculata-lessona* triploid males (Ohtani, '92).

Triploid males of *S. alburnoides*, however, seem to produce only clonal sperm as do *S. alburnoides* hybrid diploids (Alves et al., '99). Normal meiosis occurs only in *S. alburnoides* males that are nonhybrids and in tetraploids with balanced doses of both genomes (Alves et al., 2001). It must be bared in mind, however, that the number of males used in this study was very small and although likely representative of the more typical situation does not allow to entirely exclude other gametogenetic processes. The example of PAA females illustrates well the need for further investigation of the gamete formation in *S. alburnoides*: although they frequently produce haploid A eggs after exclusion of the P-genome followed by normal meiosis, the same individual may also produce diploid AA and triploid PAA gametes (Alves et al., 2004), stressing the instability of the process. The same type of instability cannot be ruled out in hybrid males with the available data. In addition, the gametogenetic behaviour of other types of males like PPA and PAAA is still awaiting investigation.

The fertility of the *S. alburnoides* males coupled with the ability of triploid females to undergo hybridogenetic meiosis is certainly an important factor contributing to the ecological success of the complex since it expands the number of possible routes to recombination and genetic variation. This scenario corroborates the point of view of several authors that highlighted the evolutionary potential of hybridization (e.g., Vrijenhoek, 2006). Indeed, instead of being necessarily ''evolutionary dead-ends'' (Dawley, '89) due to difficulties to escape the accumulation of deleterious mutations and to cope with the changing environment, hybrid complexes may experience genetic diversification that enhances their hypotheses of success (e.g., Dawley, '89; Vrijenhoek, '94; Dowling and Secor, '97; Alves et al., 2001; Stöck et al., 2002; Scali et al., 2003; Janko et al., 2005; Bi and Bogart, 2006; Gromicho et al., 2006).

In contrast with the majority of the vertebrate hybrid complexes already described, the *S. alburnoides* complex is neither a typical asexual lineage (as described by Dawley, '89) nor is it dependent on a bisexually reproducing host. Instead, this complex of minnows is constituted by both fertile males and females involved in varied reproductive modes that enhance its genetic variation, being an amazing example of the creative role that interspecific hybridization may play in evolution.

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