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Morphology, testes development and behaviour of unusual triploid males in microchromosome-carrying clones of *Poecilia formosa*

D. K. Lamatsch*†‡, M. Stöck§, R. Fuchs‖, M. Döbler*, R. Wacker*, J. Parzefall¶, I. Schlupp** and M. Schartl*

*Lehrstuhl für Physiologische Chemie I, Theodor-Boveri-Institute, Biozentrum, Am Hubland, D-97074 Würzburg, Germany, †Institute for Limnology, Austrian Academy of Sciences, A-5310 Mondsee, Austria, §Department of Ecology and Evolution, University of Lausanne, Biophore, CH 1015 Lausanne, Switzerland, ‖Department of Organismic Biology, University of Salzburg, A-5020 Salzburg, Austria, ¶Zoologisches Institut und Museum, Universität Hamburg, Hamburg, Germany and **University of Oklahoma, Department of Zoology, 730 Van Vleet Oval, Norman, OK 73019, U.S.A.

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In a microchromosome-carrying laboratory stock of the normally all-female Amazon molly *Poecilia formosa* triploid individuals were obtained, all of which spontaneously developed into males. A comparison of morphology of the external and internal insemination apparatus and the gonads, sperm ploidy and behaviour, to laboratory-bred F_1 hybrids revealed that the triploid *P. formosa* males, though producing mostly aneuploid sperm, are partly functional males that differ mainly in sperm maturation and sexual motivation from gonochoristic *P. formosa* males.

Key words: gonopodium; histology; sex determination; sperm-dependent parthenogenesis.

INTRODUCTION

The Amazon molly *Poecilia formosa* (Girard) is a unisexual species of hybrid origin (Hubbs & Hubbs, 1932; Abramoff *et al*., 1968; Avise *et al*., 1991; Schartl *et al*., 1995*a*) which reproduces by sperm-dependent parthenogenesis (gynogenesis). Allospecific sperm are needed to trigger the onset of embryogenesis but karyogamy usually does not occur. Therefore, the offspring are natural clones of the mother (Beukeboom & Vrijenhoek, 1998; Schlupp, 2005; Lampert & Schartl, 2008). The availability of sperm is an evolutionary limitation for asexual fishes forcing them into close geographic and ecological dependencies with their host, usually a sexually reproducing ancestor (Lamatsch & Stöck, 2009). Only a few species have evolved 'host switches' (*i.e*. using sperm from a non-parental species; Choleva *et al*., 2008) among them *P. formosa* (Niemeitz *et al*., 2002). It could be predicted that the

‡Author to whom correspondence should be addressed. Tel.: +43 6232 312527; fax: +43 6232 3578; email: dunja.lamatsch@oeaw.ac.at

evolution of non-sperm-dependent forms would be favoured by selection. On the other hand, rare males occurring in sperm-dependent complexes may enable the persistence of gynogenesis in absence of the host. In the study species, *P. formosa*, male-like phenotypes have been found in natural habitats, but appear to be rare (Schlupp *et al*., 1992; Lamatsch, 2001). These rare males in an usually all-female species provide a glimpse into the evolution and function of male-specific genes in a species, in which male-specific genes are no longer under stabilizing selection (Schlupp *et al*., 1998).

It is known that *P. formosa* may show masculinization to some extent. Three different types of males have been described from the *P. formosa* breeding complex. The first form is 'hormone males' (Haskins *et al*., 1960; Hamaguchi & Egami, 1980; Turner & Steeves, 1989; Schartl *et al*., 1991): newborn *P. formosa* that were exposed to male hormones pre- and post-partum develop a male phenotype. They show all secondary characteristics as well as behaviour of males, but no fully developed testis is recorded; instead ovo-testis is developed. Offspring have never been reported. The second form is 'pseudomales' or masculinized gynogens (Schlupp *et al*., 1992): diploid females that are exposed to stress (high population densities, high temperature) may spontaneously develop slightly prolonged anal fin rays 3, 4 and 5 as this is the case when males develop their copulatory organ, the gonopodium. These fish, however, are unable to move their anal fin, as during gonopodial thrusting, probably due to lack of a male-specific muscle operating the gonopodial suspensorium. Again offspring from these fish have never been reported. The third form is triploid males with microchromosomes (Lamatsch *et al*., 2000*a*): in laboratory strains which carry microchromosomes derived probably from one of the short fin mollies like *P. mexicana* or *Poecilia sphenops* Valenciennes males spontaneously occur without any apparent external trigger (*e.g*. high population densities, high temperature and skewed sex ratios). These fish show a black pigmentation pattern due to the macromelonophore locus located on the microchromosomes (Schartl *et al*., 1997). Cytogenetic and genetic analyses revealed that these males were allotriploids, which produce sperm but show irregularities in meiotic chromosome pairing (Lamatsch *et al*., 2000*a*).

In the present study, 16 triploid *P. formosa* males spontaneously occurring over a period of 9 years were investigated, corresponding to an overall frequency of considerably $<1\%$. Since there was no obvious external trigger, the aim was to understand how spontaneous male production in an 'all-female species' might occur, the evolutionary significance this might have for the breeding complex, and how these males differ from the aforementioned 'hormone males' and 'pseudomales'.

Primary and secondary sexual characters, as well as sexual behaviour in these unusual triploid *P. formosa* males were analysed and compared with artificially bred F_1 hybrids between *Poecilia latipinna* (Le Sueur) females and *Poecilia mexicana* Steindachner males for the following reasons: 1) *P. formosa* is a hybrid species (Hubbs & Hubbs, 1932; Turner *et al*., 1980; Avise *et al*., 1991; Schartl *et al*., 1995*a*), derived from the natural hybridization between a *P. mexicana* female and a *P. latipinna* male (*P. formosa* = *P. mexicana* × *P. latipinna*); 2) the triploid *P. formosa* males show the following genetic composition: [(*P. mexicana* × *P. latipinna*) + microchromosome] × *Poecilia salvatoris* Regan or *P. mexicana limantouri* (see Table I); 3) F_1 hybrids originated by crossing the two parental species *P. latipinna* and *P. mexicana*. The resulting offspring are bisexual diploid hybrids

TABLE I. Descent of triploid *Poecilia formosa* males

Male	Female stock	Father
1–6, 8, 14, 15	*Pf* 922	*Poecilia salvatoris*
7, 16	*Pf* 922	*Poecilia mexicana limantouri*
9	*Pf 637*	*P. salvatoris*
10, 12	*Pf* 533	*P. salvatoris*
11	*Pf* 1587	*P. mexicana limantouri*
13	*Pf* 537	*P. salvatoris*

(males and females; Ptacek, 2002). Some of these hybrids, however, show irregularities in meiosis which has been identified as automixis (Lampert *et al*., 2007).

Therefore, F_1 hybrid males are genetically closer to the triploid *P. formosa* males than either of the parental species. Hence, the prediction was that triploid *P. formosa* males would not strongly differ in sexual characters from sexual (hybrid) males.

In the present study, this hypothesis was tested. The results showed that the triploid *P. formosa* males did not differ significantly from gonochoristic males in morphology and mate choice towards different types of stimuli (selectivity), but do reveal pronounced differences in testes maturation, and exhibited reduced sexual activity (motivation). Due to their ability to produce sperm and trigger embryogenesis in gynogenetic females, the occurrence of similar males in natural habitats might enable *P. formosa* to become independent from their sexual hosts.

MATERIALS AND METHODS

FISHES

All fishes were raised and maintained under standard conditions as described for *Xiphophorus maculatus* (Günther) (24–27° C, 14L:10D cycle) (Kallman, 1975) in the aquarium of the Biocenter at the University of Würzburg, Germany. Twenty to 50 fishes were kept in 50 l community tanks with shared water. All phenotypic males [Fig. 1(c)–(e) and Table I] developed spontaneously in clonal lines of spotted females [Fig. 1(b)] carrying two to three microchromosomes (Schartl *et al*., 1995*b*) from matings either with *P. sphenops* males [Fig. 1(a)] or *P. mexicana* males [Fig. 1(g)]. They are derived from the following strains.

Black Amazon I (WLC 533): Animals of this clonal line exhibit a black spotted pigmentation phenotype because of the presence of a microchromosome derived from a *P. sphenops*. The founder female was from wild-type pigmented *P. formosa* strain I (WLC1357). The introgression event and origin of this line have been described in Schartl *et al*. (1995*b*). Several clonal sublines of WLC 533 were established (*e.g*. 537, 637).

Black Amazon II (WLC 922): the clonal line is similar to WLC 533, also originating from an independent introgression event of a *P. sphenops*-derived microchromosome into *P. formosa* strain I (WLC1357). Several clonal sublines of WLC 922 were established (*e.g*. 1587).

Black molly (WLC 1351): the melanistic ornamental strain is of unknown genetic origin. From body shape and mitochondrial DNA sequence, it is probably derived from the *P. mexicana* and *P. sphenops* complex (B. Wilde & M. Schartl, unpubl. data). These fishes are homogeneously dark black coloured due to the presence of macromelanophores in the skin of the body and fins. Fishes are homozygous for the dominant pigmentation loci niger (N) and melas (M) (Schröder, 1964).

Liberty mollies are an aquarium strain derived from *P. salvatoris*.

For maintaining the gynogenetic stocks, usually one male per tank per 20 to 30 *P. formosa* was used (Lamatsch *et al*., 2009).

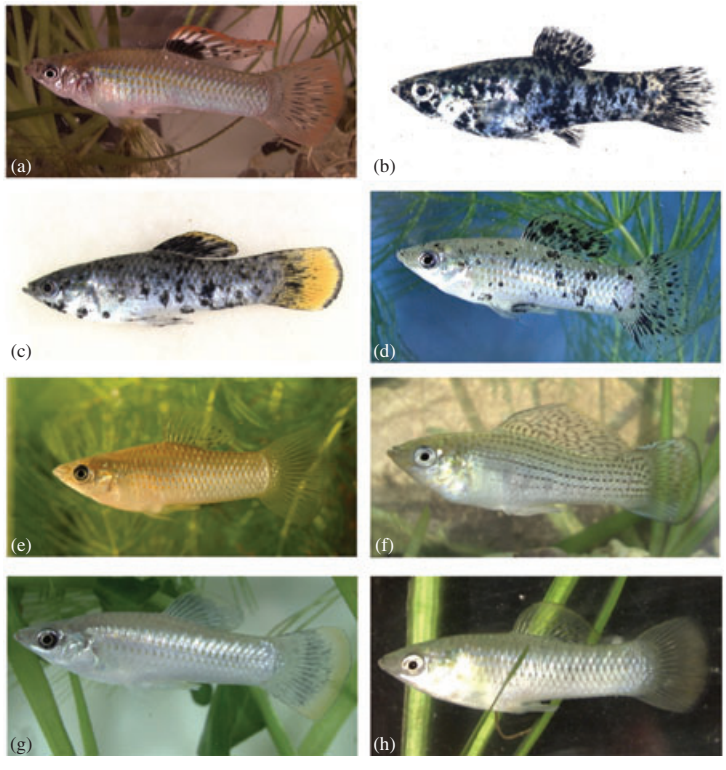

FIG. 1. Habitus of (a) a *Poecilia salvatoris* male and (b) a typical spotted female carrying microchromosomes in comparison to (c)–(e) three *Poecilia formosa* males showing different levels of pigmentation. (f)–(h) Show gonochoristic males: (f) a *Poecilia latipinna* male, (g) a *Poecilia mexicana* male and (h) a hybrid male resulting from breeding a *P. latipinna* female with a *P. mexicana* male.

The ploidy of *P. formosa* males was measured by flow cytometry (Lamatsch *et al*., 2000*b*) as soon as phenotypic changes (*e.g*. prolongation of the anal fin) were detected. Triploid status was shown for all males (Lamatsch *et al*., 2000*a*).

For comparisons of all investigated traits, F_1 hybrids (*P. latipinna* × *P. mexicana*) were used [Fig. 1(h)]. These F_1 hybrids are bisexual and fertile (Ptacek, 2002; Lampert *et al*., 2007). *Poecilia latipinna* males [Fig. 1(f)] and *P. mexicana* males [Fig. 1(g)] were used as additional control if available.

BREEDING EXPERIMENTS

A previous publication already demonstrated some sperm production in these triploid *P. formosa* males (Lamatsch *et al*., 2000*a*). Therefore, males were mated to groups of four to five spotted and wild-type coloured *P. formosa* females as well as to sexual *P. mexicana* females to test their fertility. All females were virgin as poeciliids are able to store sperm. Breeding success (production of fry) was recorded.

HABITUS AND ANATOMY OF SECONDARY SEXUAL CHARACTERISTICS

The standard length (L_S) and habitus of all males were assessed and photographed. The structure of the insemination apparatus (gonopodium, consisting of rays 3–6 of the anal fin

with the attached appendices) and the anatomy of the gonopodial suspensorium (internal anal fin rays where the male-specific muscles insert to move the gonopodium) were visualized using the Spalteholz/Dawson technique (Dawson, 1926). Briefly, the soft tissues was cleared with 3% potassium hydroxide for 3 days at room temperature, followed by the staining of bones with 0·3% alizarin red/3% potassium hydroxide, and the replacement of body fluids with glycerol (Culling, 1963). Images were taken with a Stemi SV 11 stereo microscope (Zeiss; www.zeiss.de). Five individuals of triploid *P. formosa* males were compared to five F_1 hybrid males and seven *P. mexicana* males were used as additional control.

HISTOLOGY OF TESTES

For histological examination of testes, the material was fixed for at least 5 days in 4% formaldehyde buffered in 1× PBS. Sections were routinely stained with haematoxylin and eosin (HE) and mounted in Rotihistokitt (Roth; www.carl-roth.de). Analyses were performed and images taken with an Axiophot POL microscope (Zeiss).

DNA CONTENT OF TESTIS CELLS

In order to obtain easily measurable monolayers of nuclei, fresh imprints of cross-sections of testes were prepared on microslides (Polysine adhesive). Testis imprints from a triploid *P. formosa* male and a *P. mexicana* male were prepared on the same microslide. All slides were then processed through the Feulgen reaction as described elsewhere (Klapperstück & Wohlrab, 1996; Stöck & Grosse, 1997; Stöck *et al*., 2002). Relative measurements of the integrated optical density (IOD), which is referred to as DNA content (C-value), were made with the CYDOK image analysis system (Hilgers; www.hilgers.com) at a wavelength of 546 nm. To document stages of spermatogenesis 100 testis nuclei from each male were measured. Nuclei from the testis imprints of each fish were randomly chosen by the operator on the live microscopic screen image and measured using the DNA content of somatic nuclei as standard. The DNA content of the haploid sperm nuclei of the diploid *P. mexicana* male was used as internal (*i.e*. slide-specific) relative reference value. Images were taken with Leitz DM RBE microscope (20–100×), Leica camera (www.leica.com) and Kodak Elite 200 ASA film (www.kodak.com).

BEHAVIOUR

To assess components of the sexual behaviour of unusual triploid *P. formosa* males, mate choice tests were performed and compared to the behaviour of genetically similar diploid hybrid F_1 males by presenting them pairs of females as well as a mixed male–female pair as stimuli. All possible stimulus combinations between *P. formosa*, *P. latipinna* and *P. mexicana* were used. Choice tests were performed as described in Schlupp & Ryan (1996) with slight modifications. Two different types of behavioural tests were conducted: in one test, only visual information was available to the focus individual (visual choice test) and in the other test, the fishes were allowed to interact unrestricted in the tank (full contact choice test). Stimulus fishes were chosen randomly from stock cultures (*c*. five tanks per species with *c*. 30–50 fish each) and put back after use. Each time a different stimulus pair was used to avoid possible preferences for a single stimulus fish. Each stimulus pair consisted of fishes of identical L_S (±2 mm) since larger females are usually preferred regardless of species (Gabor & Aspbury, 2008). For full contact choice tests, females were chosen to be non-receptive by eye, *i.e*. not extensively followed by a male in stock tank (Parzefall, 1973) and without an anal spot (Peden, 1973), because receptive females will always be preferred regardless of species (Schlupp *et al*., 1991).

Size ranges of fishes used in mate choice experiments are given in Appendix I. Male poeciliids stop growing as soon as they reach sexual maturity, whereas females grow lifelong (Bisazza, 1993). Therefore, females are in general larger than males. Since male mate choice was tested, the stimulus fishes were mostly female, the focus fish male. It can be seen from Appendix I, however, that triploid *P. formosa* males did not differ significantly in L_S from

hybrid males ($P > 0{\cdot}05$) and that the L_S range of stimulus fishes is within natural size distributions (Appendix I). The sequence of the stimulus pairs was randomly chosen for each male tested. Each focus male performed five visual choice tests and four full contact choice tests (plus repeated trials), unless the fish died before the trials were finished. Eleven to 12 triploid *P. formosa* males and seven diploid F_1 hybrid males were tested. Fishes were kept in a shared water system. The triploid males were raised in a group tank (25 l) with spotted and unspotted siblings. The F_1 hybrid males were raised with unspotted siblings. All males were isolated for at least 24 h prior to trials to standardize sexual motivation (Franck, 1975; Travis, 1994).

The motivation to study male mating behaviour was to understand the full impact of the masculinization observed in this study. Especially in light of the hypothesis that these males might make *P. formosa* independent of sperm donors, it is crucial to know whether or not the masculinized *P. formosa* will actually mate with conspecifics, or if their mating behaviour remains that of females, or if their behaviour is somehow confused.

In addition to full contact choice tests, visual choice tests were performed. These have the advantage of disentangling the 'male' sexual preferences from the actual interactions measured in full contact trials. While full contact trials will allow the behavioural interactions that may lead to matings (including the female response to male approaches) to be studied, the visual choice tests remove the interactive component and allow the preference only to be investigated. This is important because this probably directly reflects the effects of the spontaneous masculinizations under investigation here. These visual choice experiments were conducted as described in Schlupp *et al.* (1994) with slight modifications (see Appendix I).

In trials allowing full interaction, the male was placed with the two stimulus fishes in an 18 l tank ($41{\cdot}5 \times 28 \times 16$ cm). For acclimatization, the male was placed in a Plexiglas spawning box for 10 min followed by a 10 min observation period (Altmann, 1974) in which the behavioural elements 'following', 'nipping' and 'copulation (attempts)' (Parzefall, 1969) were recorded as 'events', and presence or absence of 'courtship behaviour' was noted. Nipping appears to aid a male in determining a female's reproductive condition (Parzefall, 1973; Travis & Woodward, 1989; Travis, 1994). In full contact choice tests, only four different trials were performed (one to four; see Appendix I). *Poecilia mexicana* male *v*. *P. mexicana* female (5) was omitted since direct contact of males would only lead to aggression behaviour. In total, seven diploid F_1 hybrid males and 12 triploid *P. formosa* males were tested.

DATA ANALYSIS

For each male type, two different aspects regarding sexual behaviour were analysed. As mentioned above, the individuals could freely choose between two different stimuli during experiments. In the different trials of the visual choice test, the times spent in front of each stimulus fish were recorded, whereas the frequency of different elements of sexual behaviour towards the different females was recorded in the full contact test (*i.e.* number of following, nipping and copulation attempts). The raw data are given in Appendices II, III and IV.

The total amount of behavioural events, *e.g.* sum of times spent in front of both stimuli fishes ($t_1 + t_2$), or total number of following, nipping and copulation attempts ($n_1 + n_2$), was used as a measure for 'motivation'. To measure 'selectivity', the difference between the two stimuli divided by the total amount of behavioural events $(t_1 - t_2)\ (t_1 + t_2)^{-1}$ was utilized, a procedure which mapped the possible values into an interval between -1 and $+1$. A value of $+1$ would mean full preference towards stimulus 1, and -1 *vice versa* for stimulus 2.

Motivational components of behaviour were tested with a repeated-measures ANOVA (visual choice) and the Friedman test (group means and full contact). For statistical analysis of selectivity, preferences towards stimuli were coded into a dichotomous (binary) variable, which could not be tested with an ANOVA design, and was therefore tested with a binomial test against a proportion of $P = 0{\cdot}5$, *i.e.* random choice of stimuli, for each trial separately. The whole sequence of trials was tested with a Cochrane Q-test. Data analysis was performed with SPSS (SPSS Inc.; www.spss.com) and R (R Development Core Team, 2008; www.r-development-core-team.software.informer.com).

RESULTS

BREEDING EXPERIMENTS

A previous publication already showed that the triploid *P. formosa* males in principle could provide the necessary stimulus to trigger parthenogenetic embryogenesis of unreduced diploid eggs in gynogenetic females due to the presence of sperm (Lamatsch *et al*., 2000*a*). To study the extent of the reproductive capacity in more detail, 16 males were individually mated with spotted and wild-type *P. formosa* females. Offspring were obtained from only five males mostly with spotted *P. formosa*. The offspring did not exhibit any signs of paternal contribution (body colouration and karyotype). No offspring were ever obtained with sexual *P. mexicana*.

HABITUS

The pigmentation of the *P. formosa* phenotypic males ranged from heavily black spotted to uniformly grey, wild-type coloured [see Fig. 1(c)–(e)]. The spotted pigmentation patterns indicated that the microchromosomes were still present (Nanda *et al*., 2007) which has also been shown by karyotyping (Lamatsch *et al*., 2000*a*). Even the darkest males, however, never reached the degree of pigmentation of normal diploid females of the same stock [Fig. 1(b)]. At sexual maturity, most of the phenotypic *P. formosa* males displayed an intensely yellow colouration of the dorsal and tail fin. In general, they had the typical body proportions of males (estimated by eye) according to Ptacek (2002). The F_1 hybrid males also showed the typical male body proportions and the yellow colour of dorsal fin and tail fin at sexual maturity (Parzefall, 1969). All phenotypic males ($n = 16$) were triploid with at least one microchromosome as determined by flow cytometry and cytogenetics, whereas all their sisters investigated were diploid ($n = 50$).

ANATOMY OF SECONDARY SEXUAL CHARACTERISTICS

The suspensoria of *P. formosa* males ($n = 5$) showed two gonapophyses and normal gonactinostal complex with baseosts [Fig. 2(a) and Table II]. The gonopodia of *P. formosa* males [Fig. 2(b)] showed all features of the structures seen in gonochoristic males (Table II). In detail, ray 3 of the gonopodia showed ventral spines and eight to 12 subdistal serrae. Ray 4 split into 4a and 4p. The 4p showed 10 to 12 double subdistal serrae and the tip of 4p was bent down. Ray 5p ended in a double bony claw in males 3, 5 and 7, and in a single bony claw in males 4 and 6. Ray 6 was shorter than rays 3, 4 and 5. Each male had a membranous hook on ray 3 and a palp of variable size. In contrast to 'hormone males' (Schlupp *et al*., 1992), every male was able to flap the gonopodium to the front (gonopodial thrusting) showing that the requisite muscles for successful insemination were developed. The triploid *P. formosa* males did not show any of the malformations of gonopodial structures which have frequently been seen in interspecies hybrids of poeciliids (Rosen & Bailey, 1963).

The diploid F_1 hybrid males ($n = 5$) also showed two gonapophyses (except one male which showed three gonapophyses) and normal gonactinostal complex

[Fig. 2(c)]. Gonopodia were normally developed [Fig. 2(d)]. One male showed a single bony claw on ray 5p in contrast to the normal double structure. Ray 4p was slightly bent down at its tip and possessed nine to 11 subdistal serrae. Ray 3 showed ventral spines, nine to 11 subdistal serrae, a membranous hook and the palp. Ray 6 was shorter than rays 3, 4 and 5 (Table II).

As in *P. mexicana* males [$n = 7$; Fig. 2(e)], all gonopodia of the triploid *P. formosa* males featured a gonopodial palp and a double bony claw on 5p (except male M1), 10 to 13 subdistal serrae on ray 4p which tip was slightly bent down. On ray 3, *P. mexicana* males showed ventral spines and eight to 11 subdistal serrae and a membranous hook. Ray 6 was shortened in comparison to rays 3, 4 and 5 [Fig. 2(f) and Table II]. In summary, triploid *P. formosa* males did not differ considerably from normal gonochoristic males in their anatomy of secondary sexual characters.

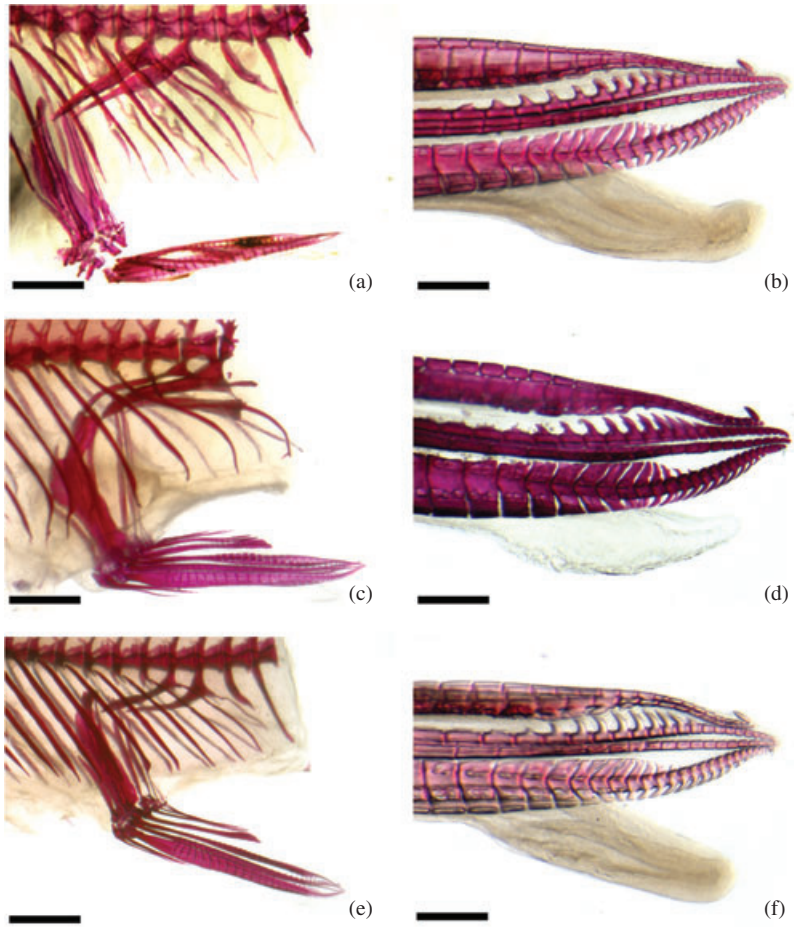

FIG. 2. (a) Suspensorium and (b) gonopodium of a triploid *Poecilia formosa* male in comparison to (c), (d) a hybrid male and (e), (f) a *Poecilia mexicana* male visualized by the Spalteholz/Dawson technique. Bar represents 2 mm for (a), (c) and (e) and 400 m for (b), (d) and (f).

TABLE II. Analysis of the variable characters of the complex insemination apparatus of triploid *Poecilia formosa* males in comparison to hybrid males and *Poecilia mexicana* males. Transformed rays of gonopodium (3–5) are numbered from bottom to top. Rays 4 and 5 are split into 4a and 4p and 5a and 5p (see also Fig. 2)

Individual	5p with bony claw	Subdistal serrae 4p	Subdistal serrae on 3	Gonapophyses
P. formosa male 3	+ (double)	11 (double)	8	2
P. formosa male 4	+ (single)	10 (double)	12	2
P. formosa male 5	+ (double)	10 (double)	9	2
P. formosa male 6	+ (single)	11 (double)	11	2
P. formosa male 7	+ (double)	12 (double)	10	2
Hybrid male 1	+ (double)	11 (double)	12	2
Hybrid male 3	+ (double)	10 (double)	11	2
Hybrid male 4	+ (single)	11 (double)	12	3
Hybrid male 5	+ (double)	9 (double)	12	2
Hybrid male 6	+ (double)	9 (double)	10	2
P. mexicana male 1	+ (double)	10 (double)	9	2
P. mexicana male 2	+ (double)	13 (double)	11	2
P. mexicana male 3	+ (double)	12 (double)	10	2
P. mexicana male 4	+ (double)	10 (double)	9	2
P. mexicana male 5	+ (double)	10 (double)	8	2
P. mexicana M1*	+ (double)	10 (double)	+	2
P. mexicana M2*	+ (single)	9 (double)	+	2

*Data from Döbler (1998); +, present.

HISTOLOGY OF TESTES

In gonochoristic *Poecilia* males, the lobules terminate at the periphery of the testis, where spermatogonia are located. Proceeding proximally, meiotic germ cells are arranged between lightly staining Sertoli cells, and primary spermatocytes, spermatids and mature spermatozoa are observed in the lumen of the testis. In contrast to hormone males which often show ovotestes (Turner & Steeves, 1989; Schartl *et al*., 1991), the gonads of the *P. formosa* males were always testis-like and showed paired morphology. Cross-sections of testes, however, showed clear differences between triploid *P. formosa* males and diploid gonochoristic males (Fig. 3). Spermatogonia and spermatocytes are present in the *P. formosa* males [Fig. 3(a)–(d)] but the proportion of mature spermatozoa and spermatozeugmata was drastically reduced in comparison to F_1 hybrid males [Fig. 3(e), (f)] or *P. latipinna* males [Fig. 3(g), (h)]. This is consistent with the observation that *P. formosa* males do not have as many offspring as gonochoristic males (Lamatsch *et al*., 2000*a*; this study).

DNA CONTENT OF TESTIS CELLS

Flow cytometric measurements of ripe sperm led to the conclusion that aneuploid sperm is produced (Lamatsch *et al*., 2000*a*). Therefore, different stages of spermatogenesis were investigated by measuring relative DNA content on fresh testis imprints. The results show that spermatogenesis of triploid *P. formosa* was

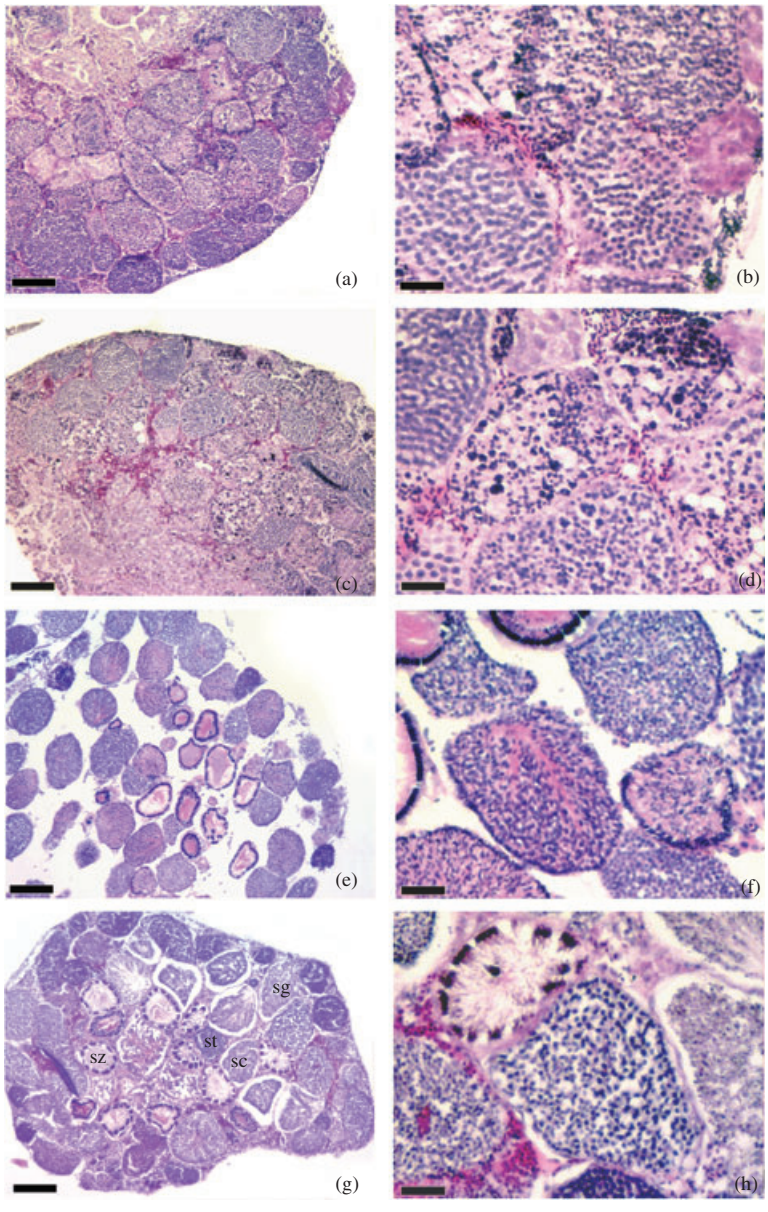

FIG. 3. Haematoxylin and eosin staining on paraffin sections of testis tissue of triploid *Poecilia formosa* male number 5 (a), (b) and number 3 (c), (d) in comparison to a (e), (f) a hybrid male and (g), (h) *P. latipinna* male. Note that ripe spermatozoa are missing from the (a)–(d) triploid *P. formosa* males. Sg, spermatogonia; Sc, spermatocytes; St, spermatids; Sz, sperm bundles. Bar represents 100 m for (a), (c), (e) and (g) and 25 m for (b), (d), (f) and (h).

highly abnormal [Fig. 4(a)]. The C-value of somatic and spermatogonial nuclei was 3C as expected and a few nuclei of 6C cells were observed in some preparations representing the somatic mitotic G2 phases. Later stages of spermatogenesis, however, showed a variety of DNA contents ranging from <0·5C to >1·8C,

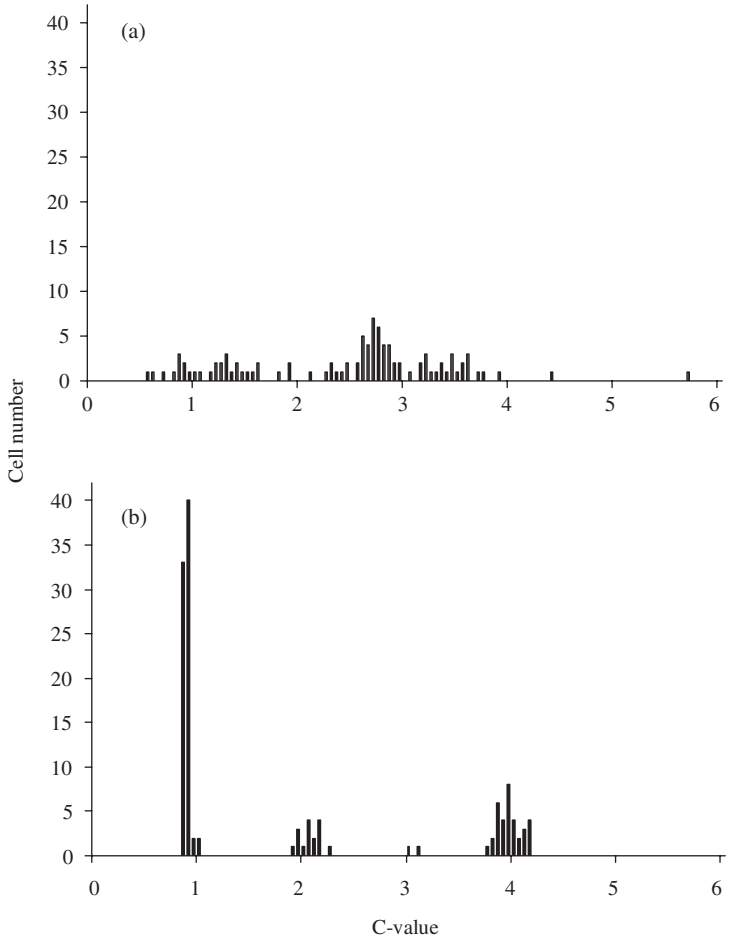

FIG. 4. Histograms of values of the relative measurements of the integrated optical density in stages of spermatogenesis in testis imprints of a (a) triploid *Poecilia formosa* male and (b) diploid *Poecilia mexicana* male.

indicating aneuploidy. Also, form and size of sperm nuclei varied much more in the triploid *P. formosa* males [Fig. 5(b), (c)] than in the diploid *P. mexicana* [Fig. 5(a)]. In *P. mexicana* males, the 2C spermatogonial cells reduplicate their DNA content and enter meiosis I in a 4C stage. After completion of meiosis I, their DNA content is 2C again and the expected haploid stage of euploid sperm is reached after meiosis II [Fig. 4(b)]. The sperm nuclei of these normal males show a distinctly more compact form and equal size [Fig. 5(a)] than of *P. formosa* males [Fig. 5(b), (c)].

BEHAVIOUR

Results for visual preference tests are given (Appendix V and Fig. A1). In addition to visual preference tests, full contact choice tests were performed giving the fishes

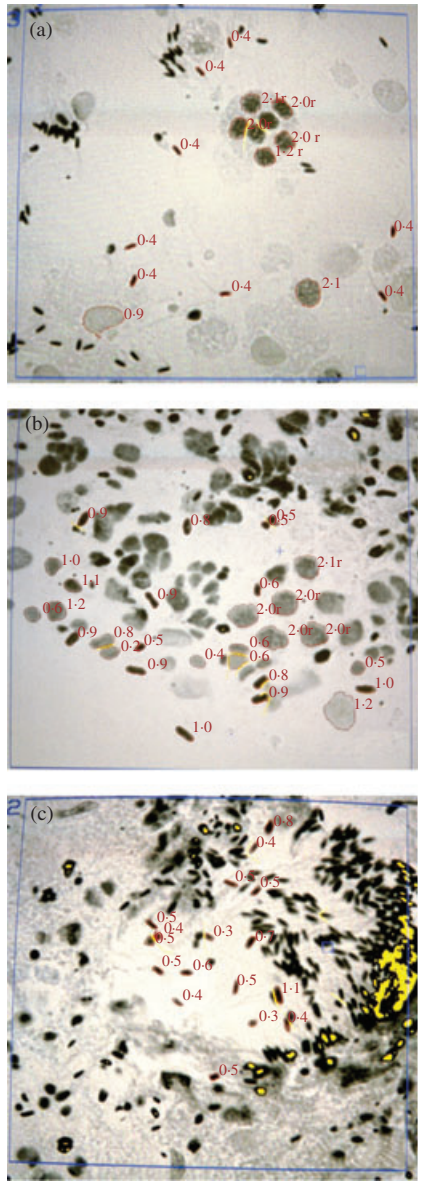

FIG. 5. Screen shots of a tissue imprint of a testis of (a) a sexual diploid *Poecilia mexicana* male in comparison with that of (b), (c) two unusual *Poecilia formosa* males. Each image shows the (relative) ratios of DNA contents as revealed by Feulgen densitometry using the image analysis system CYDOK. Somatic nuclei are used as 'reference nuclei' (always '2·0'), the DNA content of all other cells is shown as relative value. (a) 2·1/2·0: DNA content of somatic nuclei (diploid); 0·9–1·2: relative DNA content (haploid) of stages of spermatogenesis; 0·5–0·4: 'high density' sperm nuclei as reached after completion of spermiohistogenesis. Note the almost constant value of the sperm DNA content. (b) 2·0: DNA content of somatic nuclei (triploid); 0·4–0·9: 'high density' sperm nuclei as reached after completion of spermiohistogenesis. Note the large range of value of the sperm DNA content suggesting aberrant and aneuploid sperm. (c) 2·0: DNA content of somatic nuclei (triploid; outside the screen); 0·3–1·1: 'high density' sperm nuclei as reached after completion of spermiohistogenesis. Note the large range of value of the sperm DNA content suggesting aberrant and aneuploid sperm.

TABLE III. Choice preferences of diploid F_1 hybrid males and triploid *Poecilia formosa* males concerning the three behavioural elements measured in the full contact tests: (a) following, (b) nipping and (c) copulation (attempts) (see Appendix III)

Trial	Stimulus	*n* (diploid)	Binomial *P*	*n* (triploid)	Binomial *P*	*n* (all)	Binomial *P*
(a) Following							
1	*Poecilia formosa*	2	>0·05	4	>0·05	6	>0·05
	Poecilia latipinna	5		6		11	
2	*P. formosa*	2	>0·05	5	>0·05	7	>0·05
	Poecilia mexicana	5		5		10	
3	*P. latipinna*	4	>0·05	8	>0·05	12	>0·05
	P. mexicana	3		2		5	
4	Spotted	0	<0·05	3	>0·05	3	<0·05
	Unspotted	7		7		14	
Cochran Qd.f. = 3		7	>0·05	10	>0·05	17	>0·05
(b) Nipping							
1	*P. formosa*	5	>0·05	5	>0·05	10	>0·05
	P. latipinna	2		3		5	
2	*P. formosa*	2	>0·05	4	>0·05	6	>0·05
	P. mexicana	5		4		9	
3	*P. latipinna*	3	>0·05	4	>0·05	7	>0·05
	P. mexicana	4		3		7	
4	Spotted	0	<0·05	3	>0·05	3	<0·05
	Unspotted	7		5		12	
Cochran Qd.f. = 3		7	<0·05	5	>0·05	12	<0·05
(c) Copulation (attempts)							
1	*P. formosa*	4	>0·05	4	>0·05	8	>0·05
	P. latipinna	3		0		3	
2	*P. formosa*	2	>0·05	1	>0·05	3	>0·05
	P. mexicana	4		3		7	
3	*P. latipinna*	4	>0·05	2	>0·05	6	>0·05
	P. mexicana	3		3		6	
4	Spotted	0	<0·05	2	>0·05	2	<0·05
	Unspotted	7		34		10	
Cochran Qd.f. = 3		6	>0·05	2	>0·05	8	<0·05

the opportunity not only to perceive visual signals but also chemical and tactile cues, corresponding to other important sensory systems in fishes (Franck & Geissler, 1973). Again, diploid F_1 hybrids showed a higher motivation to perform different types of sexual behaviour (see Fig. 6 and Table III). Testing the groups' means with the Friedman test yielded significant differences concerning the number of nipping and copulations attempts (both d.f. = 1, $P < 0{\cdot}05$), whereas the difference in following behaviour was not significant ($P > 0{\cdot}05$; Table IV).

There were no significant differences in choice between the two groups in trials 1–3, but the clear preference of diploid F_1 hybrids for wild-type mates in trial 4 (Fig. 6). Accordingly, no copulation attempt towards a spotted female was

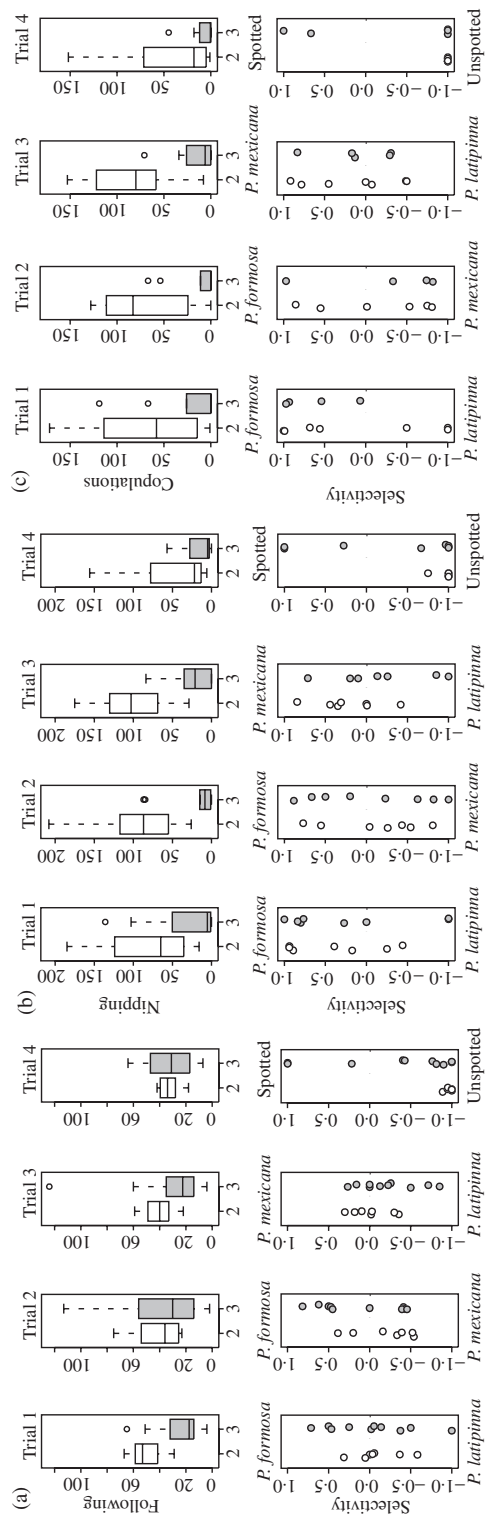

FIG. 6. Full contact choice tests. Results of four different experimental set-ups recording three different elements of mating behaviour for diploid F_1 hybrid males (2) and triploid *Poecilia formosa* males (3) within a 10 min observation interval: (a) following, (b) nipping and (c) copulation (attempts). Total numbers of behavioural elements are shown in upper rows and resemble 'motivation'. The corresponding rows below show selectivity towards different stimuli in these tests (see Fig. A1). Whisker = 1·5 × interquartile range.

TABLE IV. Group means of the total numbers of the three behavioural elements measured in full contact tests between diploid F_1 hybrid males (2) and triploid *Poecilia formosa* males (3) resembling motivation. Corresponding test results of the Friedman test between both groups are given below

Trial	Ploidy	Following	Nipping	Copulation
1 *P. formosa v. Poecilia latipinna*	2	49·9	83·6	69·7
	3	25·2	34·6	22·7
2 *P. formosa v. Poecilia mexicana*	2	41·9	95·0	69·0
	3	38·0	22·2	13·8
3 *P. latipinna v. P. mexicana*	2	40·7	100·9	86·0
	3	34·3	26·1	16·6
4 Spotted *v.* unspotted	2	32·7	52·4	46·3
	3	32·8	16·0	7·9
Friedman test (d.f. = 1)	*P*-value	>0·05	<0·05	<0·05

recorded [Fig. 6(c)], and only one diploid male nipped only once on a spotted female [Fig. 6(b)]. In contrast to Ptacek (2002), neither hybrid males nor *P. formosa* males showed courtship display.

DISCUSSION

In most of the unisexual vertebrate complexes, rare phenotypic males have been described, *e.g.* in *Darevskia* lizards (Darevski *et al*., 1978, 1986; Kupriyanova, 1989), as well as in teleosts such as *Carassius gibelio* (Bloch) (Abramenko *et al*., 1998), *Squalius alburnoides* (Steindachner) (Alves *et al*., 1999), *Phoxinus eos-neogaeus* (Cope) (Goddard & Dawley, 1990), *Cobitis* sp. (Vasil'ev *et al*., 2003) and *Misgurnus anguillicaudatus* (Cantor) (Morishima *et al*., 2004; Itono *et al*., 2006). Their occurrence does raise several questions: Are these males functional? If so, does their occurrence have consequences for the breeding complex? Are these phenomena linked to sex determination and dispensable genes?

Here, allotriploid males in the unisexual species *P. formosa* are described, in order to evaluate their functionality (physiologically and behaviourally), and therefore their potential evolutionary significance for the breeding complex. Besides the pigmentation pattern, there were no striking differences between the triploid *P. formosa* males and the F_1 hybrid males in morphological traits. *Poecilia formosa* males showed the typical male phenotype, including body proportions as well as yellowish pigmentation of the fins. The expression of the microchromosome-specific pigmentation locus, however, changes the overall appearance of these males, which might have an effect on female mate choice. The pigmentation pattern was always less pronounced than in their diploid mothers. The expression of the pigmentation gene of the microchromosome may be differentially modified by the presence or absence of a third chromosome set as shown by Pala *et al*. (2008) for gene expression in allotriploid *S. alburnoides*.

All *P. formosa* males possessed a fully differentiated suspensorium and gonopodium. The number of subdistal serrae of ray 4p varied within as well as

between the different male types and deviated slightly from those reported by Dramsch (1977) or Döbler (1998). Showing all features of the anatomy of the complex insemination apparatus of poeciliids, it is inferred, however, that triploid *P. formosa* males can be classified as functional males.

Although triploid *P. formosa* males show the typical testis morphology, they differ strikingly from normal males in the number of mature spermatozoa. Analysing DNA content and ploidy level of testis cells by IOD revealed a reduced number of 6C cells, as well as aneuploid sperm. The data may be explained by the fact that after the G2 of meiosis daughter cells with aneuploid numbers of chromosomes are formed resulting in aneuploid sperm. As a result, spermatogenesis is impaired, meiosis arrested at S2 and most cysts in triploid males remain in the spermatogonial and spermatocyte stages. Because of this difficulty of equally distributing three chromosome sets in meiosis, sexual reproduction of triploids is usually impossible. This is corroborated by the fact that no offspring were obtained with sexual *P. mexicana* females but only with *P. formosa* females for which chromosome balance does not play a pivotal role for only activating embryonic development.

In the behavioural choice tests neither of the two different male types showed a significant preference for females of either species. Given the choice between a male and a female, however, each male type preferred the male. Therefore, the comparison of the two types of males revealed no significant difference. Sexual selection involves two main mechanisms: intrasexual competition for mates (intermale competition) and intersexual mate choice. Balsano *et al*. (1985) demonstrated that dominant *P. mexicana* males direct 63% of their activities to other males, and only 37% to females. As the *P. formosa* males and F_1 hybrid males have been kept solitarily during the daily tests, it was assumed that they showed (probably sexual) dominance behaviour towards males. This is an important finding that could not be achieved by full contact tests.

The only significant difference between the triploid *P. formosa* males and the diploid F_1 hybrid males was found in the trial of spotted *v*. wild-type *P. formosa*. The F_1 hybrid males spent significantly more time in front of the wild-type female and interacted more with it. *Poecilia formosa* males, however, did not choose clearly between the two female types but tended to prefer the spotted females. This interpretation gets further support from the fact that the triploid *P. formosa* males predominantly produced offspring with spotted *P. formosa* females. Landeau & Terborgh (1986) and Theodorakis (1989) showed that phenotypically different individuals are prone to higher predation risk (oddity effect). Therefore, given the choice, an individual should decide to shoal with similar individuals, a pattern which McRobert & Bradner (1998) and Ledesma & McRobert (2008) were able to show by studying bright and dark *P. latipinna* (Blakeslee *et al*. 2008; Gómez-Laplaza, 2009). Whether this is the case also in choosing a mating partner is not clear. Another possible explanation could be that the males chose a familiar stimulus. Since the triploid *P. formosa* males, in contrast to the diploid F_1 hybrid males, had been raised with their spotted as well as unspotted (= wild-type) sisters, experience could have influenced the mate choice (Magurran *et al*., 1994; Körner *et al*., 1999). This has to be studied more intensively.

Overall, no significant differences in the selectivity of *P. formosa* males and F_1 hybrid males were found. This was cautiously interpreted as absence of major differences. Clearly the analysis is limited through small sample sizes but, as shown in the preference test of spotted *v*. wild-type *P. formosa*, differences can be resolved even with this small sample number. Thus, the findings indicate that the *P. formosa* males described here are capable of typical male behaviour and should be viewed as functional males in this respect. They showed, however, a reduced motivation to perform different types of sexual behaviour (*i.e.* total presenting time, total number of following, nipping and copulation attempts) in comparison to F_1 hybrid males. The lower overall sexual activity of *P. formosa* males may be due to lower androgen levels, but may also reflect a loss of genes coding for male characteristics. Different studies have shown that 11-ketotestosterone (11-KT) plays an important role for triggering the male sexual behaviour (Borg, 1987; Brantley *et al*., 1993). Oligospermy in combination with lower sexual motivation might therefore be the reason for the low breeding success of the triploid *P. formosa* males (only five males out of 16 sired offspring).

Sex steroids are classically known to operate through two distinct mechanisms to affect physiology and behaviour in vertebrates: organizational (dimorphic differentiation of brain morphology during ontogeny) and activational (effects on fully developed nervous system) (Cooke *et al*., 1998). The 'selectivity component' should reflect the organizational effect (*i.e.* development) of the brain. Since there is just one clear difference in 'selectivity' in the test 'spotted *v*. unspotted' (possibly simply because diploid fish were not habituated to spotted fish), it seems that male-specific imprinting of the brain has taken place during ontogeny of the triploid *P. formosa* males. The high and consistent difference in frequency of the according behaviour, however, points towards lower activational mechanisms (*i.e.* actual hormone level).

In rare cases, phenotypic males of *P. formosa* have been observed in natural habitats (Hubbs *et al*., 1959; Hubbs, 1964; Darnell & Abramoff, 1968; Döbler, 1998). Their ploidy and presence of microchromosomes, however, was never investigated, and in some cases their hybrid character cannot be ruled out (Schlupp *et al*., 1992). Provided that the *P. formosa* males occurring in nature are also triploid and have similar properties to those described in the present paper (triploid with microchromosome), the intriguing question arises whether their inadequate meiotic performance, presumably resulting in aneuploid sperm, may still have some evolutionary significance under the special rules of gynogenesis. In Lamatsch *et al*. (2000*a*) and this study, it is shown that aneuploid sperm is able to function quite adequately as a trigger in gynogenesis. If this is true, gynogenetic lineages may sometimes become at least partly independent from their sexual hosts. Despite their low frequency under laboratory conditions, conclusions about frequencies in nature cannot be drawn. Natural populations of *P. formosa*, however, are large, therefore increasing the probability to obtain males. Unfortunately, males found in nature have not been quantitatively and qualitatively investigated. A survey of natural habitats for males and subsequent investigation of their karyotype is underway (I. Schlupp, pers. comm.).

Despite their potential evolutionary significance, the question of what are the sex-determining factors leading to these unusual triploid males in an otherwise all-female species cannot be answered yet. Is it the additional chromosome set or the

macromelanophore locus-containing microchromosome (Schartl *et al*., 1997) or the combination of both? In *X. maculatus*, it has been shown that gene loci involved in different pigmentation patterns and in sexual maturity are closely linked to the sex-determining locus in the subtelomeric region of the X- and Y-chromosomes (Kallman, 1984; Gutbrod & Schartl, 1999). The presence of the microchromosome alone, however, is unlikely to explain the occurrence of males since none of the diploid spotted individuals has ever developed a male phenotype. In addition, the third chromosome set alone is not sufficient to cause a sex reversal since all unspotted triploids detected were females (Monaco *et al*., 1984; Nanda *et al*., 1995; Lamatsch *et al*., 2000*b*, 2004). Therefore, the combination of both, an additional chromosome set and the macromelanophore locus-containing microchromosome, are probably necessary for the male development. To what extent the microchromosome and the third genome influence the sex determination in an otherwise all-female species will be the subject of future studies.

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Appendix I. Standard length (L_S) mean ± s.d. and range of fishes used in behavioural mate choice experiments

	Poecilia formosa male	Hybrid male	*Poecilia mexicana* male	*Poecilia latipinna* male	*Poecilia formosa* female	*P. mexicana* female	*P. latipinna* female
	Focus fishes				Stimulus fishes		
Mean ± s.d. L_S (mm)	34·00 ± 6·32	30·29 ± 2·05	32·39 ± 3·53	31·20 ± 3·76	35·62 ± 2·86	35·28 ± 2·99	35·06 ± 2·69
n	8	7	28	15	97	61	54
L_S range (mm)	24–44	27–34	26–38	24–38	28–45	29–45	29–42
				Fishes from natural habitats			
Mean ± s.d. L_S (mm)	—	—	32·30 ± 5·69	28·20 ± 6·12	41·30 ± 8·80	36·22 ± 5·05	37·20 ± 5·80
n	—	—	10	935	949	95	1016
L_S range (mm)	—	—	20–40	16–53	30–67	28–54	30–61

n, sample size.

Visual choice tests: in addition to full contact choice tests, visual choice tests were performed. These have the advantage of disentangling the male sexual preferences from the actual interactions measured in full contact trials. While full contact trials will allow study of the behavioural interactions that may lead to matings (including the female response to male approaches), the visual choice tests remove the interactive component and allow investigation of the preference only. This is important because this probably directly reflects the effects of the spontaneous masculinizations under investigation here. Visual choice experiments were conducted in a tank (71·5 × 28 × 16 cm) that was divided into five sections. The two end sections, separated by clear partitions, contained the stimulus fishes. The middle section was the neutral zone. The two sections adjacent to the stimuli were defined as preference zones (Kodrik-Brown, 1989), and sojourn of the focus fish in each of the preference zones was recorded in seconds. For each trial, a test male was placed in a Plexiglas cylinder in the neutral zone of the tank and the stimulus fishes were placed into the side compartments. After 5 min of acclimatization time, the Plexiglas cylinder was carefully removed, and the time the test fish spent in each preference zone during a 5 min trial was recorded. To avoid side biases, the side assignment of stimuli was swapped and the trial repeated with the same stimulus pair. A side bias was *a priori* defined as a test fish spending >80% of its time on the same side of the tank (independent of the position of the stimuli) which leads to exclusion of this trial (Schlupp *et al*., 1994). Trials yielding reaction indices (calculated as time spent in preference zones per 600 s) of <66% were also excluded (Schlupp *et al*., 1994). In both cases, the experiment was repeated later with the same individual but with a different stimulus pair, because males were first not motivated to choose between the presented stimuli. Five different stimulus combinations were tested: 1) *P. formosa v. P. latipinna*, 2) *P. formosa v. P. mexicana,* 3) *P. latipinna v. P. mexicana*, 4) *P. formosa* spotted *v. P. formosa* unspotted (= wild-type) and 5) *P. mexicana* male *v. P. mexicana* female. In total, seven F_1 hybrid males and 11–13 *P. formosa* males were tested.

APPENDIX II. Raw data on visual choice tests. Time (s) spent next to stimulus fish: (a) triploid *Poecilia formosa* males and (b) diploid F_1 hybrid males. Unless stated differently stimulus fish are female

	Stimulus pair 1		Stimulus pair 2		Stimulus pair 3		Stimulus pair 4		Stimulus pair 5		Stimulus pair 6	
	P. formosa	*Poecilia latipinna*	*P. formosa*	*Poecilia mexicana*	*P. mexicana*	*P. latipinna*	Spotted	*P. formosa*	*P. mexicana* male	*P. mexicana*	*P. latipinna* male	*P. latipinna*
(a) *Poecilia formosa* male												
1	61	438	446	74	211	280			395	107		
3	278	158	247	213	195	253	346	212	236	196		
4	269	197	295	164	285	151	438	67	389	94		
5	107	417	57	453	167	301	538	15	307	233		
6			169	196	161	389	249	281	499	49		
7	281	242	371	190	306	199	227	306	274	197		
8	131	445	60	488	534	43	50	506	444	132		
9	189	364	146	356	299	181	256	234	313	197	257	168
10					558	0						
11	252	256	299	193	216	212	102	417	322	232	414	121
12	264	276	270	237	85	484	258	279	182	316	29	498
15	342	192	355	103	111	414	226	285	295	234	209	292
16	411	172	147	337	66	422	272	144	379	165	0	576
Mean ± S.D.	235 ± 99	287 ± 110	239 ± 119	250 ± 131	246 ± 148	256 ± 146	269 ± 130	250 ± 142	336 ± 85	179 ± 74	182 ± 153	331 ± 200

Appendix II. Continued

	Stimulus pair 1		Stimulus pair 2		Stimulus pair 3		Stimulus pair 4		Stimulus pair 5		Stimulus pair 6	
	P. formosa	*Poecilia latipinna*	*P. formosa*	*Poecilia mexicana*	*P. mexicana*	*P. latipinna*	Spotted	*P. formosa*	*P. mexicana* male	*P. mexicana*	*P. latipinna* male	*P. latipinna*
(b) Hybrid male												
1	29	543	156	398	202	347	68	508	357	190	411	141
2	297	194	350	127	469	89	7	578	374	146	597	0
3	154	401	417	133	275	274	0	597	594	0	322	214
4	329	208	150	429	375	181	112	450	422	116	323	181
5	127	459	507	59	584	2	22	538	570	27	599	0
6	165	414	186	368	294	245	59	539	35	543	597	0
7	73	493	60	535	208	362	20	559	504	85	506	80
Mean ± s.d.	168 ± 102	387 ± 136	261 ± 152	293 ± 183	344 ± 131	214 ± 133	41 ± 37	538 ± 49	408 ± 174	158 ± 182	479 ± 117	88 ± 92

Results on visual preference tests: when only visual cues were perceptible, triploid *P. formosa* males spent less time in front of both stimuli fish than did diploid F_1 hybrid males (RM-ANOVA, between-subjects effect, d.f. = 1, $P = 0{\cdot}001$). On the other hand, there were no significant differences in 'motivation' (total presenting time) in both groups between the five different trials [within-subject effects, Huyn-Feldt, d.f. = 3·52, $P > 0{\cdot}05$; interaction term: $P > 0{\cdot}05$; Fig. A1 (a)].

Additionally, there were no differences between the two groups, regarding the selectivity towards the different stimulus species given in trials 1–3, where no preference for a particular species could be detected [Fig. A1 (b)]. In trial 4, diploid F_1 hybrids clearly showed a preference for unspotted fish, *i.e.* wild-type habitus [Fig. A1 (b)]. Both groups showed a preference for males in trial 5 [binomial test, $n = 19$, $P = 0{\cdot}001$; Fig. A1 (b) and Appendix V].

Testing for differences in preference between all five trials with the Cochrane Q-test showed a significant result (d.f. = 4, $P < 0{\cdot}01$), due to selectivity of diploid fishes for wild-type fishes in trial 4 and those of both groups for males in trial 5 (see Appendix V for details).

APPENDIX III. Raw data on full contact choice tests of *Poecilia formosa* males. Total numbers of (a) following, (b) nipping and (c) copulation attempts. Unless stated differently stimulus fishes are female

	Stimulus pair 1		Stimulus pair 2		Stimulus pair 3		Stimulus pair 4	
P. formosa male	*P. formosa*	*Poecilia latipinna*	*P. formosa*	*Poecilia mexicana*	*P. mexicana*	*P. latipinna*	Spotted	Unspotted
(a) Following								
3	0	3	1	0	0	0	400	0
4	12	4	20	2	2	25	14	33
5	0	0	1	1	0	2	1	0
6	25	26	15	36	13	22	1	19
7	12	2	3	1	1	3	2	15
8	1	3	4	10	2	12	10	0
9	22	8	47	11	7	7	39	25
11	20	12	28	10	9	9	3	29
12	28	37	17	39	38	22	0	30
13	0	5	1	1	7	9	57	0
15	5	11	82	31	72	52	0	44
16	9	10	6	16	12	19	2	5
Mean ± S.D.	11·2 ± 10·4	10·1 ± 10·9	18·8 ± 24·3	13·2 ± 14·4	13·6 ± 21·1	15·2 ± 14·4	44·1 ± 113·5	16·7 ± 15·6
(b) Nipping								
3	0	3	0	0	0	0	300	0
4	9	1	5	1	2	25	1	5
5	0	0	0	0	0	0	0	0
6	25	25	5	8	13	22	0	3
7	1	0	0	0	0	0	0	0
8	0	1	0	0	0	1	4	0
9	125	11	1	10	0	0	23	13

Appendix III. Continued

	Stimulus pair 1		Stimulus pair 2		Stimulus pair 3		Stimulus pair 4	
P. formosa male	*P. formosa*	*Poecilia latipinna*	*P. formosa*	*P. mexicana*	*P. mexicana*	*P. latipinna*	Spotted	Unspotted
11	28	16	9	6	13	17	1	56
12	91	12	16	69	38	31	0	28
13	0	1	3	1	9	6	23	0
15	0	0	82	5	72	12	0	3
16	0	0	0	1	0	0	0	0
Mean ± s.d.	23·3 ± 41·5	5·8 ± 8·3	10·1 ± 23·2	8·4 ± 19·4	12·3 ± 21·8	9·5 ± 11·5	29·3 ± 85·7	9·0 ± 16·9
(c) Copulation (attempts)								
3	11	1	5	0	0	0	0	4
4	0	0	0	0	0	0	0	0
5	0	1	0	0	0	0	400	0
6	8	7	0	0	9	17	0	0
7	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0
9	115	4	1	10	0	0	15	3
11	20	6	2	4	13	10	0	45
12	66	1	7	47	12	22	0	3
13	0	0	0	0	7	5	12	0
15	0	0	66	1	65	6	0	1
16	0	0	0	0	0	0	0	0
Mean ± s.d.	18·3 ± 35·8	1·7 ± 2·5	6·8 ± 18·8	5·2 ± 13·5	8·8 ± 18·4	5·0 ± 7·6	35·6 ± 114·9	4·7 ± 12·8

APPENDIX IV. Raw data on full contact choice tests of F_1 hybrid males. Total numbers of (a) following, (b) nipping and (c) copulation attempts. Unless stated differently stimulus fishes are female

	Stimulus pair 1		Stimulus pair 2		Stimulus pair 3		Stimulus pair 4	
Hybrid male	*Poecilia formosa*	*Poecilia latipinna*	*P. formosa*	*Poecilia mexicana*	*P. mexicana*	*P. latipinna*	Spotted	Unspotted
(a) Following								
1	25	28	6	20	27	28	1	17
2	23	12	12	24	13	9	0	25
3	14	53	11	35	19	40	1	38
4	24	24	52	23	28	15	1	41
5	14	15	7	16	17	18	0	34
6	30	27	15	10	17	14	0	40
7	19	41	26	36	14	26	0	31
Mean ± S.D.	21·3 ± 5·9	28·6 ± 14·3	18·4 ± 16·2	23·4 ± 9·5	19·3 ± 6·0	21·4 ± 10·6	0·4 ± 0·5	32·3 ± 8·8
(b) Nipping								
1	7	18	6	20	54	54	0	49
2	63	2	10	17	30	73	0	6
3	27	19	94	27	44	17	1	7
4	6	10	77	10	71	6	0	107
5	175	10	8	75	76	77	0	156
6	164	5	100	108	118	57	0	22
7	55	24	32	81	19	10	0	19
Mean ± S.D.	71·0 ± 70·8	12·6 ± 8·0	46·7 ± 42·3	48·3 ± 38·8	58·9 ± 33·2	42·0 ± 30·3	0·1 ± 0·4	52·3 ± 57·7
(c) Copulation (attempts)								
1	1	3	0	0	37	43	0	37
2	58	0	1	7	25	73	0	1
3	21	4	93	27	41	5	0	3
4	0	1	38	3	68	3	0	106
5	171	1	8	75	73	73	0	152
6	0	160	63	65	111	42	0	18
7	53	15	24	79	2	6	0	7
Mean ± S.D.	43·4 ± 61·4	26·3 ± 59·2	32·4 ± 35·0	36·6 ± 35·4	51·0 ± 35·9	35·0 ± 31·0	0·0 ± 0·0	46·3 ± 59·3

APPENDIX V. Visual choice preferences of the two different groups of males (diploid F_1 hybrid males, triploid *Poecilia formosa* males) and overall values are shown for five different stimulus pairs in the visual choice test. *P*-values of the corresponding binomial tests are given to show significant differences in stimulus choice within each trial. Differences in choice between the trials were tested with the Cochran Q test. [Different sample sizes (*n*) appear because two triploid fish did not perform all different trials due to sudden death.]

Trial	Stimulus	*n* (diploid)	Binomial *P*	*n* (triploid)	Binomial *P*	*n* (all)	Binomial *P*
1	*P. formosa*	2	>0·05	5	>0·05	7	>0·05
	Poecilia latipinna	5		6		11	
2	*P. formosa*	3	>0·05	7	>0·05	10	>0·05
	Poecilia mexicana	4		5		9	
3	*P. latipinna*	2	>0·05	7	>0·05	9	>0·05
	P. mexicana	5		5		10	
4	Spotted	0	<0·05	5	>0·05	5	>0·05
	Unspotted	7		6		13	
5	Male	6	>0·05	11	<0·01	17	0·001
	Female	1		1		2	
Cochran Q		7	<0·01	10	>0·05	17	<0·01

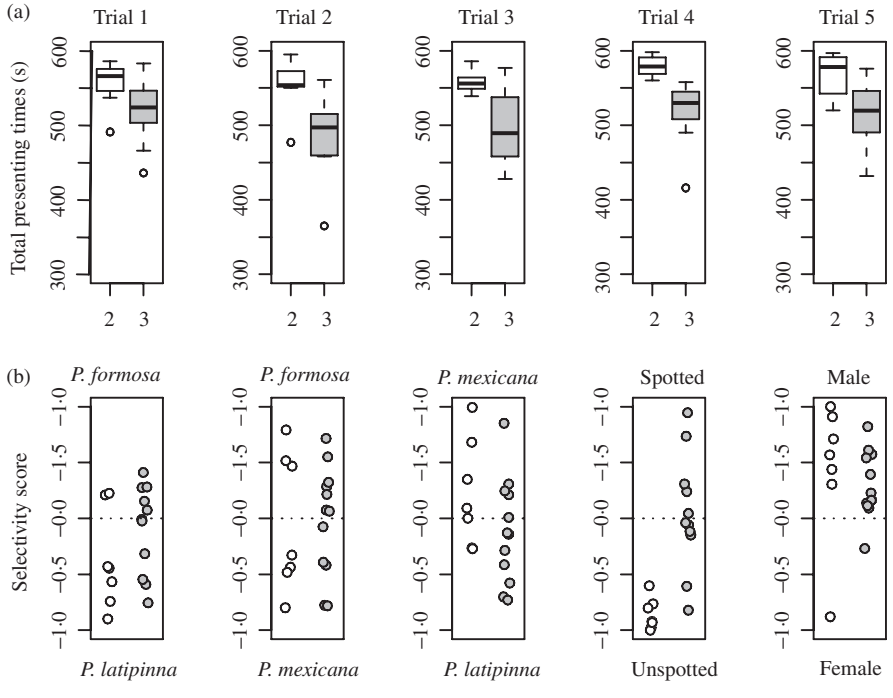

FIG. A1. Results of five visual choice trials with different rivalling stimuli are presented. (a) The total times ($\sum t$) of presenting behaviour within a 10 min observation interval, serving as a measure for motivation of the two different groups of males (2/white = diploid F_1 hybrid male, 3/grey = triploid *Poecilia formosa* male). (b) The corresponding selectivity score ($\Delta t \sum t^{-1}$) of the individual fish in these trials. Whisker = 1·5 × interquartile range.