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Spontaneous polyploidy, gynogenesis and androgenesis in second generation (F_2) koi *Cyprinus carpio* × goldfish *Carassius auratus* hybrids

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The objective of this study was to characterize the genetics of second generation (F_2) koi *Cyprinus carpio* × goldfish *Carassius auratus* hybrids. Spermatozoa produced by a novel, fertile F_1 male were found to be diploid by flow-cytometric analysis. Backcross (F_1 female × *C. carpio* male and *C. carpio* female × F_1 male) juveniles were triploid, confirming that female and male F_1 hybrids both produced diploid gametes. The vast majority of surviving F_2 juveniles was diploid and small proportions were aneuploid (2·1*n*–2·3*n* and 3·1*n*–3·9*n*), triploid (3*n*) and tetraploid (4*n*). Microsatellite genotyping showed that F_2 diploids repeated either the complete maternal or the complete paternal genotype. Fish with the maternal genotype were female and fish with the paternal genotype were male. This demonstrates that F_2 diploids were the result of spontaneous gynogenesis and spontaneous androgenesis. Analysis of microsatellite inheritance and the sex ratio in F_2 crosses showed that spontaneous gynogenesis and androgenesis did not always occur in equal proportions. One cross was found to have an approximate equal number of androgenetic and gynogenetic offspring while in several other crosses spontaneous androgenesis was found to occur more frequently than spontaneous gynogenesis.

Key words: aneuploid; common carp; interspecies hybrid; microsatellite; tetraploid.

INTRODUCTION

The viability and fertility of F_1 interspecies fish hybrids are frequently affected by the presence of two different haploid chromosome sets. In many cases, these chromosome sets are incompatible for meiosis and the hybrids are sterile (Chevassus, 1983). In some cases, interspecies hybrid females have been found to produce mass triploid progeny when backcrossed to males of their parental species (Ojima *et al.*, 1975; Dawley *et al.*, 1985; Johnson & Wright, 1986), indicating that the female hybrids produce diploid eggs. This was demonstrated in the case of Prussian carp *Carassius gibelio* (Bloch 1782) × common carp *Cyprinus carpio* L. 1758 hybrid females by Cherfas *et al.* (1994) and in the case of ornamental *C. carpio* (koi) × goldfish *Carassius auratus* L. 1758 hybrids by Gomelsky *et al.* (2012). The reproductive biology of

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Carassius spp. × *C. carpio* hybrids has been investigated by several different groups and reviewed by Gomelsky (2015).

A series of studies into interspecies *C. gibelio* (bisexual form) × *C. carpio* hybrids performed in Russia from 1977 to 1992 is detailed in Cherfas *et al.* (1994). It was found that while some F_1 hybrid females were fertile, all F_1 hybrid males were sterile. Backcrosses of the hybrid females to *C. gibelio* and *C. carpio* males yielded triploid progenies. It was concluded that the hybrid females produced diploid eggs; the mechanism was shown to be premeiotic endoreduplication during oogenesis (Emelyanova & Cherfas, 1980; Emelyanova, 1984).

Cherfas *et al.* (1994) reported that several successive gynogenetic generations were produced by inseminating eggs from hybrid females with genetically inactivated sperm. Tissue transplantation studies and analysis of polymorphic proteins both indicated that hybrid gynogens originating from one fish were genetically identical to each other. Several gynogenetic progenies were sex-reversed by the application of androgens and these sex-reversed hybrid males (XX) were fertile, unlike genotypic males (XY). When the sex-reversed males were backcrossed to *C. carpio* females, the offspring were triploid. It was suggested that the sex-reversed male hybrids produced diploid spermatozoa (Gomelsky *et al.*, 1988).

The production of diploid eggs and diploid spermatozoa made it possible to produce tetraploid hybrids. Gomelsky *et al.* (1988) reported that during two spawning seasons sex-reversed hybrid males and hybrid females were crossed. In both years, the progenies consisted of two distinct size groups. When karyotyped, the group of large fish was found to be composed of diploids and the group of small fish was composed almost entirely of tetraploids. The appearance of diploids in the progenies was proposed to be the result of spontaneous gynogenesis and androgenesis.

Liu *et al.* (2001) detailed the results of long term studies of *C. auratus* (bisexual form) × *C. carpio* hybrids performed in China. F_1 hybrids produced haploid eggs and spermatozoa and by normal crossing diploid F_2 hybrids were produced. F_2 hybrids produced diploid eggs and spermatozoa and further crosses produced mass tetraploid progeny in the third hybrid generation (F_3). The tetraploid hybrids produced diploid gametes and further generations of tetraploids, up to F_8, were produced. Xiao *et al.* (2013) reported that F_{22} had successfully been produced and that all generations between F_3 and F_{22} were tetraploid. The production of diploid gametes by tetraploids demonstrates that tetraploidy restores normal meiosis with the reduction of chromosome number from the somatic to the gamete level in *C. auratus* × *C. carpio* hybrids. These findings differ in part from Cherfas *et al.* (1994) and Gomelsky *et al.* (2012) where both groups reported that F_1 hybrid females produce diploid eggs and from Cherfas *et al.* (1994), who found no fertile F_1 male hybrids, although sex-reversed males were fertile.

Wu *et al.* (2003) described a separate series of studies on hybrids between *C. carpio* and *C. auratus* (bisexual form) in China. In the F_1 hybrid generation, all males were found to be sterile, but the female hybrids were fertile and produced diploid eggs. Triploid hybrids were produced by backcrossing the females with males from both parental species. Triploid females were found to be fertile and when these females were crossed with males of the parental species, triploid offspring were produced. The offspring were found to be morphologically identical to the maternal triploid females suggesting that the triploid females produced unreduced triploid eggs that developed gynogenetically. In the progenies produced by crossing a triploid

hybrid female with a *C. auratus* male, a small number of male and female tetraploids were found, suggesting that occasionally the maternal and paternal pronuclei fuse and develop normally (Wu *et al.*, 2003). When the eggs from the female tetraploids were fertilized by active or inactive sperm, all-female, tetraploid progenies were produced. Analysis of microsatellite marker inheritance demonstrated that the tetraploid females were propagating *via* natural gynogenesis (Ye *et al.*, 2002, 2009).

In summation, the production of both reciprocal crosses of *Carassius* spp. × *C. carpio* hybrids has been reported by several groups, but each group reported different observations. Given these differences, further investigation into these hybrids is needed in order to better understand the mechanisms involved.

Cherfas *et al.* (1994) noted that research into *Carassius* spp. × *C. carpio* hybrids could help to explain how several complexes of fishes, all of which have an interspecies hybrid origin, changed from sexual reproduction to either gynogenesis or another mechanism referred to as hybridogenesis. These complexes of fishes were recently reviewed in depth by Lamatsch & Stöck (2009). One example is the *Cobitis elongatoides–taenia* complex, which contains diploid, triploid and tetraploid forms that reproduce gynogenetically (Vasil'ev *et al.*, 1989; Saat, 1991). These fish are the result of relatively recent hybridization events between two spined loaches *Cobitis elongatoides* Băcescu & Mayer 1969 and *Cobitis taenia* L. 1758 (Janko *et al.*, 2003). An ancient hybridization event between the Iberian chub *Squalius pyrenaicus* (Günther 1868) and a now extinct ancestor led to the development of the Iberian roach *Squalius alburnoides* (Steindachner 1866) complex, which includes diploid, triploid and tetraploid forms (Alves *et al.*, 2001). Triploid females contain one chromosome set from *S. pyrenaicus* and two chromosome sets from the unknown ancestor. These triploids exhibit a reproductive mode known as meiotic hybridogenesis, which entails the elimination of the *S. pyrenaicus* chromosome set followed by a normal meiosis between the two remaining chromosome sets from the unknown ancestor and results in a haploid egg. Triploid females breed with diploid males and the offspring develop into diploid individuals. The majority of diploid females resulting from these crosses produce unreduced diploid eggs that develop into triploid individuals after fertilization with a normal haploid sperm (Alves *et al.*, 1998). Naturally occurring triploid pond loach *Misgurnus anguillicaudatus* (Cantor 1842) females, which are in the same family (Cobitidae) as the *Cobitis* spp. previously discussed, have also been found to produce haploid eggs through meiotic hybridogenesis (Morishima *et al.*, 2008). These three examples, *Cobitis* spp., *M. anguillicaudatus* and *Squalius* spp., of natural gynogenetic and hybridogenetic reproduction illustrate that interspecies hybridization can be an important trigger for the development of asexual reproductive modes observed in several teleosts.

As mentioned above, it has recently been shown in studies performed in the U.S.A. that F_1 female *C. carpio* × *C. auratus* hybrids produce diploid eggs (Gomelsky *et al.*, 2012). Using microsatellite markers, it has been demonstrated that these eggs contain the entire maternal genome (Anil *et al.*, 2016). F_1 hybrid males at Kentucky State University have previously been found to be sterile (Gomelsky *et al.*, 2012). In 2012, however, one fertile F_1 hybrid male was found at Kentucky State University making the production of F_2 hybrid progenies possible (B. Gomelsky, unpubl. data). The objective of this study was to produce F_2 and backcross (F_B) *C. carpio* × *C. auratus* hybrids and investigate their ploidy, inheritance of microsatellite markers and sex segregation.

MATERIALS AND METHODS

ORIGIN OF ANALYSED PROGENY, FISH REARING AND FISH BREEDING

This study was carried out at the Aquaculture Research Center of Kentucky State University in Frankfort, Kentucky, U.S.A. F_1 broodstock were produced at Kentucky State University as part of earlier investigations by crossing *C. carpio* females with *C. auratus* males. The *C. auratus* males were obtained from a local baitfish farm and thus belong to the single domesticated lineage (Rylková *et al.*, 2010). All *C. carpio* and *C. auratus* broodstock were tested for ploidy by flow cytometry and shown to be diploid (Gomelsky *et al.*, 2012). Eight progeny groups were raised and analysed for ploidy and sex segregation (Table I). Groups 1–3 were F_2 progenies obtained by crosses of three separate F_1 females with the one fertile F_1 male and were also analysed for microsatellite inheritance. F_1 Female 1, the dam for group 1, displayed the Design trait, a pigmentation pattern characteristic of metallic koi (a variety of ornamental *C. carpio*). This trait is controlled by a dominant allele of a single gene (*D*/*d*) (Katasonov, 1973; Kirpitchnikov, 1999) and segregation of this trait was observed and analysed in group 1. Groups 4 and 5 were F_2 progenies obtained by crossing two separate F_1 females with the fertile F_1 male. Groups 7 and 8 were backcrosses (F_B) obtained by spawning the same two F_1 females (used for groups 4 and 5) with two separate *C. carpio* males. Group 6 was also a backcross (F_B) progeny, but it was obtained by crossing a *C. carpio* female with the fertile F_1 male.

All progeny groups were produced by artificial spawning. Spawning was induced by injecting carp pituitary extract (Argent Laboratories; www.argent-labs.com) in doses of 3 mg kg^{-1} body mass. The females were given a 10% priming injection and a 90% resolving injection, separated by 12 h. The males were given the full dosage in one injection *c.* 6 h after the females were given the priming injection. Eggs and sperm were collected by stripping and the dry method of fertilization was used. Two minutes after fertilization, the eggs were treated with a 1:8 volumetric cow milk:water solution and stirred for 1 h to prevent adhesiveness. The fertilized eggs were then transferred to McDonald jars and incubated until hatching of larvae. Swim-up larvae were collected in hapas placed in a raceway and then transferred to 20 m^3 outdoor tanks for rearing for 5 months. In the autumn, the number of surviving juveniles was counted, samples of fish were weighed and the fish were transferred to indoor recirculating systems for 6 months before dissection and analysis of sex segregation. Water temperature in the recirculation systems was maintained at 25° C.

SEX IDENTIFICATION OF FISH

The sex of fish was determined by dissection and observation of gonad morphology. Based on the descriptions of Gupta (1975) and Smith & Walker (2004), the 11 month old fish were identified as male or female. Gupta (1975) describes the gross morphology of both the testes and the ovaries in developing *C. carpio* and Smith & Walker (2004) detail the gross morphology of the developing ovaries. Using the noted differences in gross morphology between *C. carpio* testes and ovaries, the developing fish were easily identified as male or female.

MICROSATELLITE ANALYSIS

Fin clips were taken from juveniles in groups 1–3 and preserved in 95% ethanol. There were 40 diploid juveniles from group 1, 30 diploid juveniles each from groups 2 and 3 and 11 aneuploid juveniles from groups 1 and 2 that were analysed. Genomic DNA was extracted from the samples according to the Wizard Genomic DNA Purification Kit (Promega; www.promega.com) mouse tail protocol, with the exception that the proteinase K digestion step was carried out at 65° C instead of 55° C. A Genequant Pro spectrophotometer (GE Healthcare-Life Sciences; www.gelifesciences.com) was used to measure the concentration of the extracted DNA. The extracted DNA was diluted to a 10 μg mL^{-1} working solution to use as the template for PCR.

Primers for five microsatellite markers used successfully in *C. carpio*, *C. auratus* and *C. carpio* × *C. auratus* hybrids by Anil *et al.* (2016) were chosen for use and were custom synthesized with a FAM fluorophore on the forward primer (Integrated DNA Technologies;

TABLE I. Origin, survival, sex distribution and ploidy of *Cyprinus carpio* × *Carassius auratus* hybrid progenies

Number and type of progeny group	Female	Male	Number of larvae stocked	Number of juveniles collected	Percent juveniles collected	Sex distribution		Ploidy distribution of juveniles (%)					
						Number of males	Number of females	Number of juveniles analysed	Euploid			Aneuploid	
									$2n$	$3n$	$4n$	$2{\cdot}1n-2{\cdot}3n$	$3{\cdot}1n-3{\cdot}9n$
1 (F_2)	F_1-1	F_1	1500	79	5·3	21	19	71	87·3	0	0	0	12·7
2 (F_2)	F_1-2	F_1	4000	123	3·1	30	0	102	97·1	0	0	0	2·9
3 (F_2)	F_1-3	F_1	500	68	13·6	30	0	37	97·3	0	0	0	2·7
4 (F_2)	F_1-4	F_1	1610	171	10·6	32	2	34	94·7	0	0	5·3	0
5 (F_2)	F_1-5	F_1	12 180	100	0·8	26	8	46	80·9	4	7	3·4	4·7
6 (F_B)	*C. carpio*	F_1	2500	2000	80	20	0	20	0	100	0	0	0
7 (F_B)	F_1-4	*C. carpio*	1330	1225	92·1	23	17	15	0	100	0	0	0
8 (F_B)	F_1-5	*C. carpio*	5320	2728	51·3	21	19	15	0	100	0	0	0

https://www.idtdna.com/). The targeted loci were *Mfw7*, *Mfw13*, *Mfw26*, *Cca02* and *Koi105-106*. Loci *Mfw7*, *Mfw13* and *Mfw26* were described in *C. carpio* by Crooijmans *et al.* (1997). Locus *Koi105-106* was described in *C. carpio* by David *et al.* (2001). Locus *Cca02* was described in *C. carpio* by Yue *et al.* (2004). After genotyping the broodstock, only loci *Mfw26*, *Koi105-106* and *Cca02* were found to differ between the male and female broodstock and so the juveniles were only genotyped for these three loci.

The targeted loci were first amplified by PCR. The PCR was performed using an Applied Biosystems Veriti thermal cycler (Applied Biosystems; www.appliedbiosystems.com). One reaction consisted of 20 ng DNA template, 0·2 μM forward and reverse primers, 0·25 U *Taq* DNA polymerase, 1·5 mM $MgCl_2$, 0·20 mM of each deoxynucleotide triphosphate (dNTP), 1× Colorless GoTaq Flexi Buffer and diH_2O for a volume of 10 μl. The PCR profile was initial denaturation at 94° C for 3 min followed by 35 cycles of denaturation at 94° C for 30 s, annealing at a primer specific temperature for 1 min, extension at 72° C for 1 min and then one final extension at 68° C for 5 min.

The PCR product was denatured at 95° C in formamide to obtain single stranded DNA. The reaction products were imaged by fluorescent capillary electrophoresis on an ABI 3500 Genetic Analiser (Applied Biosystems) with GeneScan 500 ROX (ThermoFisher Life Technologies; www.thermofisher.com) as the size standard. GeneMapper 3.5 (Applied Biosystems) was used to analyse the data and report the length of the amplified fragments.

FLOW-CYTOMETRY ANALYSIS OF FISH PARENTS AND JUVENILES

Determinations of ploidy were made using measurements of nuclear DNA content obtained by flow cytometry. Blood samples were taken from the juveniles and broodstock immediately before analysis, as well as from several largemouth bass *Micropterus salmoides* (Lacépède 1802) for use as an internal control. The samples were drawn from the caudal vein into 3·0 ml Vacutainer tubes containing lithium heparin. One µl of sample blood and 0·5 μl of *M. salmoides* blood were added to 500 μl Propidium Iodide Staining Solution (Biosure; www.biosure.com). The samples were mixed by inversion and incubated in the dark at room temperature for 10 min. An Accuri C6 (BD Biosciences; www.bdbiosciences.com) flow cytometer was used to record 40 000 events from each sample. Nuclear DNA content was determined by comparing the intensity of the fluorescence peak from the sample to the intensity of the fluorescence peak from the internal control. Ploidy was calculated by comparing the measured DNA content of each sample with the average measured DNA content of several diploid *C. carpio*.

In groups 1–3 and 7–8, the fish selected for ploidy analysis were sampled at random. It was observed that in groups 1–3 the aneuploids were significantly smaller than the diploids. In order maximize the chance of detecting aneuploid juveniles, in groups 4 and 5 the smallest 10% of fish were selected for screening and then a random sample was taken from the remaining 90% of fish. The ploidy frequency distributions in these two groups were calculated by adjusting for the applied sampling procedure by weighting the observations according to the proportion of the population they represent.

FLOW CYTOMETRY OF SPERMATOZOA FROM F_1 HYBRID MALE

Measurements of the ploidy of spermatozoa were performed in a method similar to the protocol for flow cytometric ploidy analysis of milt described by Peruzzi *et al.* (2009). Milt was collected from the fish by manual stripping. One μl of milt, along with 0·5 μl of *M. salmoides* blood as an internal control, was added to 500 μl Propidium Iodide Staining Solution (Biosure). The samples were incubated in the dark at room temperature for 10 min and then 40 000 events were recorded for each sample using an Accuri C6 flow cytometer (BD Biosciences). Nuclear DNA content was determined by comparing the intensity of the fluorescence peak from the sample to the intensity of the fluorescence peak from the internal control. Ploidy was calculated by comparing the measured DNA content of each sample with the average measured DNA content of milt from several diploid *C. carpio*.

TABLE II. Microsatellite genotypes of F_1 parents and F_2 juvenile diploids

Progeny group	Microsatellite locus	Parental genotypes		Number of fish analysed	F_2 diploids	
		Female	Male		Maternal genotype[a]	Paternal genotype[b]
1	*Mfw26*	119/154	106/154	40	19	21
	Koi105-106	148/175	170/175		18	21
	Cca02	175/175	177/177		19	21
2	*Mfw26*	123/140	106/154	30	0	30
	Koi105-106	153/183	170/175		0	30
	Cca02	176/176	177/177		0	30
3	*Mfw26*	112/140	106/154	30	0	30
	Koi105-106	170/192	170/175		0	30
	Cca02	157/157	177/177		0	30

[a]Fish with maternal microsatellite genotype were all females.
[b]Fish with paternal microsatellite genotype were all males.

RESULTS

The number of stocked larvae, number of surviving juveniles and survival percentage for all groups raised are shown in Table I. Survival was low in all five F_2 groups, ranging from 0·8 to 13·6% with an average of 6·7%. In contrast, survival in the three F_B groups was much higher, ranging from 51·3 to 92·1% with an average of 74·5%. Ploidy analysis of each F_2 group revealed that the vast majority of juveniles were diploid, but small proportions of aneuploids were found in F_2 groups 1–5. Most of the aneuploids found had ploidy values in the range of $3{\cdot}1n$–$3{\cdot}9n$. Group 5 was composed of 7% tetraploid juveniles and these fish displayed normal external morphology. Ploidy analysis of the three backcross groups revealed that all backcross juveniles were triploid. Sex segregation varied among the F_2 groups, with group 1 having approximately equal numbers of males and females and groups 2–5 having more males than females. In the two F_1 hybrid female × *C. carpio* backcross groups, groups 7 and 8, there were *c.* equal numbers of males and females, while in the *C. carpio* × F_1 hybrid male backcross group, group 6, all fish were male. Spermatozoa produced by the fertile F_1 male were diploid and no spermatozoa of other ploidy levels were detected. Spermatozoa from the control *C. carpio* males were haploid.

The microsatellite genotypes of the breeders and F_2 diploid juveniles from groups 1 to 3 are shown in Table II. The diploids from group 1 were found to repeat either the paternal genotype at all three loci or the maternal genotype at all three loci. The only exception was one fish which repeated the maternal genotype at two loci, but no amplification of the *Koi105-106* locus was detected. In groups 2 and 3, all diploids had genotypes identical to the paternal genotype at all three loci. No mixing of paternal and maternal alleles was observed in any diploid juvenile. The sex of each diploid analysed for microsatellite genotype was determined. All fish with the paternal microsatellite genotype were males and all fish with the maternal microsatellite genotype were females. In group 1, all fish with the maternal microsatellite genotype displayed the Design trait while all fish with the paternal microsatellite genotype did not.

Eleven aneuploid F_2 juveniles from groups 1 and 2 were also analysed for microsatellite genotype (Table III). These aneuploid fish were found to have inherited both

TABLE III. Microsatellite genotypes of F_2 juvenile aneuploids (for parental genotypes, see Table II)

Fish	Progeny group	Ploidy (*n*)	Microsatellite genotype		
			Mfw26	*Koi105-106*	*Cca02*
1	1	3·5	106/119/154	148/170/175	175/177
2	1	3·4	106/119/154	148/170/175	175/177
3	1	3·7	106/119/154	148/170/175	175/177
4	1	3·3	106/119/155	148/170/175	175/177
5	1	3·4	106/154	148/170/175	175/177
6	1	3·4	106/119/154	148/170/175	175/177
7	1	3·4	106/119/154	170/175	175/177
8	1	3·9	106/119/154	148/170/175	175/177
9	2	3·2	106/123/154	153/170/175	176/177
10	2	3·4	106/123/154	170/175/183	176/177
11	2	3·3	106/154	153/170/175	176/177

maternal and paternal microsatellite alleles. Most fish displayed three different alleles at loci *Mfw26* and *Koi105-106*.

DISCUSSION

Of all the F_1 males produced by the hybridization of *C. carpio* with *C. auratus* at Kentucky State University, only the one described in this study produced spermatozoa. It is unclear why this male was fertile and the others were not. The production of mass triploids in group 6, a backcross progeny of a *C. carpio* female to this male, demonstrates that this male produced diploid spermatozoa. Direct measurements of spermatozoa ploidy by flow cytometry confirmed this and showed that spermatozoa of other ploidy levels were not present in detectable numbers. Cherfas *et al.* (1994) reported that genotypic male (XY) hybrids were sterile, while sex reversed male (XX) hybrids were fertile and so it was first considered that this fertile male was the result of spontaneous sex reversal. Based on group 6 being composed entirely of males and the presence of males in groups 1–5 (F_2 groups), however, it can be deduced that the fertile F_1 male described in this study is a genotypic male (XY).

All analysed backcross juveniles from groups 7 and 8 were triploid, further supporting the conclusions of Gomelsky *et al.* (2012) that F_1 hybrid females produce diploid eggs.

The ploidy distributions of surviving juveniles from groups 1 to 5 (Table I), all obtained by $F_1 \times F_1$ hybridization, demonstrate that surviving juvenile F_2 hybrid populations are composed almost entirely of diploid fish with small proportions of aneuploid and tetraploid fish also present. It is unclear why more tetraploid juveniles were not observed, as the fertilization of diploid eggs with diploid spermatozoa should result in tetraploid embryos. It is possible that tetraploids have very low viability and while the majority of embryos and larvae could be tetraploid, most are not able to survive until the end of the growout season. The low survival observed in all F_2 progeny groups is consistent with this suggestion.

In contrast to this study's observations, Gomelsky *et al.* (1988) reported that tetraploid hybrids were viable, but significantly smaller than diploids produced in

the same cross, resulting in bimodal size distributions. All tetraploids were found to be female and to sexually mature. When the tetraploids were crossed with *C. carpio* males, the resulting progeny were triploid, indicating that the tetraploid females produced diploid eggs and thus that normal meiosis was restored with the reduction of chromosome number from the somatic to the gamete level. A group of tetraploids were sex reversed by the application of androgens. It was found that these sex reversed males were fertile and backcrossing these males to *C. carpio* females resulted in triploid progenies. This indicates that the sex-reversed male tetraploids produced diploid spermatozoa. Cherfas *et al.* (1994) reported that the crossing of tetraploid female hybrids with tetraploid sex-reversed male hybrids produced a second generation of all female tetraploid hybrids. Liu *et al.* (2001) were also able to produce viable tetraploids for many consecutive generations. It is unclear why in the current study, while there were potentially many tetraploid embryos because both F_1 females and the F_1 male produced diploid gametes, surviving tetraploid juveniles were present in small numbers and in only one group of fish.

The genetic origin of diploids in the F_2 generation was successfully determined through the use of microsatellite DNA markers (Table II). All diploids were found to repeat either the complete maternal or paternal genotype over all tested loci, thus proving them to be of gynogenetic or androgenetic origin. The sex of the diploids was found to correspond to the genotype that was inherited. Fish inheriting the maternal genotype were female and fish inheriting the paternal genotype were male. In species which display male heterogamety, such as *C. auratus* (Yamamoto & Kajishima, 1968) and *C. carpio* (Gomelsky, 2015), induced androgenetic progenies are composed of both males and females because diploidy is restored by suppression of the first mitotic division, creating XX females and YY males (Bongers *et al.*, 1999). In this case of spontaneous androgenesis, the microsatellite data demonstrates the lack of classical segregation and so all diploid spermatozoa were presumably XY causing all androgens to be XY males. Additionally, in group 1 the Design trait was present in all of the female diploids but none of the male diploids. This strengthens the conclusion that F_2 diploids are the result of spontaneous gynogenesis and androgenesis. The one fish, which displayed no amplification at locus *Koi105-106*, displayed the maternal genotype at the other two examined loci, was female and displayed the Design trait. This female is likely the result of spontaneous gynogenesis and the absence of an allele is probably due to a failure in the amplification process.

In group 1, both androgens and gynogens were found in approximately equal numbers. In groups 2 and 3, however, only androgens were found and in groups 4 and 5 the ratio was heavily skewed towards androgens. It can be concluded that in some F_2 hybrid crosses spontaneous androgenesis occurs more frequently than spontaneous gynogenesis. This suggests that the female pronucleus is spontaneously inactivated more frequently than the male pronucleus, although the mechanism by which the inactivation occurs is unclear. Since the proportion of androgens was found to vary between progenies and all the progenies had the same sire, it is likely that the ratio of spontaneous androgenesis to spontaneous gynogenesis in a given progeny is dependent on a maternal genetic factor.

The appearance of aneuploid juveniles in F_2 hybrid progenies has not been previously reported. While they made up a small proportion of the juvenile population, it is possible that aneuploids made up a larger proportion of the larvae and many did not survive to the juvenile stage. These aneuploids could be the result of abnormalities

occurring during meiosis, such as chromosome non-disjunction. Microsatellite analysis of the few surviving aneuploid juveniles revealed that they inherited alleles from both the dam and the sire (Table III), demonstrating that they are the result of successful fertilization and karyogamy.

Gomelsky *et al.* (1988) reported that in progenies produced by crossing *C. gibelio* × *C. carpio* hybrid females with hybrid sex-reversed males, diploids were present even though both the hybrid females and the hybrid sex reversed males produced diploid gametes. Gomelsky *et al.* (1988) suggested that these diploids were the result of spontaneous androgenesis and gynogenesis. The current study makes use of microsatellite markers to confirm this suggestion and demonstrate that the origin of F_2 diploids was spontaneous androgenesis and gynogenesis.

Diploids have been observed to occur in the offspring of other fishes that produce diploid gametes and it has been suggested that this is the result of spontaneous androgenesis and gynogenesis. Chourrout *et al.* (1986) report the results of breeding tetraploid rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) males that produced diploid spermatozoa. In progenies produced by crossing the tetraploid males with diploid females, the majority of embryos were triploid, but a small proportion of embryos were diploid. Chourrout *et al.* (1986) suggested that the diploid embryos were of androgenetic origin and would presumably be viable since they were diploid. Johnson & Wright (1986) reported the results of crossing F_1 brown trout *Salmo trutta* L. 1758 × Atlantic salmon *Salmo salar* L. 1758 hybrid females with male *S. salar*. The F_1 hybrid females produced diploid eggs and in the backcross progenies both triploid and diploid fish were produced. Isozyme analysis was performed on these fish and it was found that the diploid fish had identical isozyme profiles to the maternal F_1 hybrids, indicating that the diploids were the result of spontaneous gynogenesis. Galbreath & Thorgaard (1995) crossed F_1 *S. trutta* × *S. salar* hybrid females with *S. salar* males and all surviving juveniles were triploid hybrids, reinforcing that these hybrid females produce diploid eggs. Galbreath *et al.* (1997) reported performing induced gynogenesis by fertilizing eggs from F_1 *S. trutta* × *S. salar* hybrid females with UV irradiated *O. mykiss* sperm but not applying any shocks to the eggs. The fertilized eggs developed into diploid *S. trutta* × *S. salar* hybrids and DNA fingerprinting with multilocus oligonucleotide probes showed that fish within each progeny were genetically identical to each other and to the maternal parent. This demonstrated that the diploid hybrid eggs contained a copy of the maternal genome that was capable of developing gynogenetically. In summary, the occurrence of spontaneous androgenesis and gynogenesis when crossing fishes that produce diploid gametes has been suggested by several groups but not proven. The current study makes use of microsatellite markers to prove that this hypothesis is correct.

Anil *et al.* (2016) reported using microsatellite markers to demonstrate that diploid eggs produced by *C. carpio* × *C. auratus* hybrids contained the entire maternal genome, with no recombination of alleles occurring during meiosis. These results are consistent with premeiotic endomitosis being the mechanism by which diploid eggs arise, as shown by Emelyanova & Cherfas (1980) and Emelyanova (1984). The spontaneous androgens identified in the current study repeated the paternal microsatellite genotype, showing no recombination of alleles at the three tested microsatellite loci during meiosis in the fertile F_1 hybrid male. These observations demonstrate the lack of classical segregation and suggest that an exact copy of the paternal genome is present in each diploid spermatozoon, which is inherited as a whole *via* spontaneous androgenesis. It is very probable that, just as is the case for female hybrids, premeiotic endomitosis is

the mechanism responsible for the production of diploid gametes with no new genetic variation by this male F_1 hybrid. This suggestion, however, should be confirmed by cytogenetic studies.

The fertility and reproductive biology of F_1 and F_2 *C. carpio* × *C. auratus* hybrids could have important implications for the control of invasive *C. carpio* and *C. auratus* in locations where both species are present and hybridization is occurring, such as Lake Mead (Goodbred *et al.*, 2013) and the Little Calumet River (Mahon *et al.*, 2013) in the U.S.A. and the Murray–Darling Basin in Australia (Haynes *et al.*, 2012). In addition to this current study, several other studies have examined these hybrids and obtained a variety of results (Cherfas *et al.*, 1994; Liu *et al.*, 2001; Wu *et al.*, 2003). Depending on the fertility of further generations and whether or not tetraploids can develop into a viable, reproducing strain of fish, these hybrids could become an additional invasive species and add to the damage already being done by invasive *C. carpio* and *C. auratus*. Future research should therefore be directed at assessing the fertility of subsequent generations of hybrids.

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