Failure of interspecies androgenesis in salmonids

I. Babiak*, ††, S. Dobosz†, H. Kuzminski†, K. Goryczko†, ‡, S. Ciesielski§, P. Brzuzan§, B. Urbányi†‡, Á. Horváth¶, F. Lahnsteiner AND J. Piironen**

*Department of Animal Biochemistry, University of Warmia and Mazury, 10-957 Olsztyn, Poland, †Department of Salmonid Research, Inland Fisheries Institute, Rutki, 83-330 Zukowo, Poland, ‡Department of Fish Biology and Culture, University of Warmia and Mazury, 10-957 Olsztyn, Poland, §Department of Evolutionary Genetics, University of Warmia and Mazury, 10-957 Olsztyn, Poland, ¶Szent István University, Department of Fish Culture, H-2103 Gödöllő, Hungary, †Institute of Zoology, Salzburg University, A-5020 Salzburg, Austria and **Finnish Game and Fisheries Research Institute, Saimaa Fisheries Research and Aquaculture, 58175 Enonkoski, Finland

(Received 31 May 2001, Accepted 21 June 2002)

Androgenetic development of salmonid embryos was induced in recipient oocytes from the same or other species (intra- or interspecies androgenesis). Parameters for induced androgenesis were investigated in brown trout Salmo trutta and brook trout Salvelinus fontinalis. Reciprocal androgenetic and control crosses were conducted among fishes from three genera: Oncorhynchus (rainbow trout, O. mykiss), Salmo (brown trout) and Salvelinus (brook trout), and within two genera: Salmo (brown trout and Atlantic salmon, S. salar) and Salvelinus (brook trout and Arctic char, S. alpinus). Live hatched androgenetic progenies were obtained in all intraspecies variants, where oocytes and sperm originated from the same species. Interspecies androgenesis resulted in no viable larvae, despite the fact that most hybrid controls and intraspecies androgenetic individuals were viable. When recipient oocytes originated from other genera (interspecific intergeneric androgenesis), embryonic development ceased in early developmental stages, except for haploid controls of brook trout produced in eggs of brown trout. Survival of embryos to the eyed-egg stage was relatively high in the intrageneric androgenesis experiment. Nevertheless, none of these embryos survived to hatching. Some of the presumed Atlantic salmon individuals developing in brown trout eggs contained maternal DNA, questioning the accuracy of enucleation using irradiation. The inability to induce interspecific androgenesis among the examined salmonid species may have been the result of substantial kariotypical and developmental differences between spermatozoal donors and oocyte recipients, causing an incompatibility between maternal cytoplasmic regulatory factors and the paternal nuclear genome.

Key words: androgenesis; embryo; fish; hybrid; reproduction; trout.

INTRODUCTION

Androgenesis, the induced development of individuals inheriting exclusively the paternal nuclear genome, has been accomplished in only a few fish species (Ihssen et al., 1990; Pandian & Koteeswaran, 1998). The difficulties in producing viable androgenetic progeny result from the drastic treatments applied to oocytes before insemination (maternal nuclear DNA eliminated by irradiation) and to
zygotes at the first mitotic division (thermal or pressure shock for doubling the haploid chromosome set). Aside from the production of homozygous and clonal lines and of monosex populations using spermatozoa from androgenetic YY ‘supermales’, androgenesis is considered a reliable tool for the recovery of entire fish cryopreserved genome information (Thorgaard, 1986; Thorgaard & Cloud, 1993).

Most studies on androgenesis have concerned salmonids of the genus *Oncorhynchus*. Few of the studies have reported survival high enough to establish androgenetic stocks (Parsons & Thorgaard, 1985; Scheerer et al., 1986, 1991; Thorgaard et al., 1990; Nagoya et al., 1996; Babiak et al., 2002). Scheerer *et al.* (1991) first demonstrated that androgenetic rainbow trout *Oncorhynchus mykiss* (Walbaum) are able to produce gametes and that androgenetic development from cryopreserved spermatozoa is possible. Recently, Babiak *et al.* (2002) established a stock of androgenetic rainbow trout produced using cryopreserved spermatozoa. This shows promise for genome banking programmes in rainbow trout, however the technology is not developed in other salmonid species. Apart from *Oncorhynchus*, successful androgenesis has also been induced in brook trout *Salvelinus fontinalis* (Mitchell) (May *et al*., 1988). No data are reported for *Salmo*.

Interspecific androgenesis (induced androgenetic development in oocytes originating from other species) is considered as a means to restore extinct taxons from their cryopreserved spermatozoa as well as to investigate nucleocytoplasmic compatibility (Bercsenyi *et al*., 1998). In salmonids, early developmental stages are controlled exclusively by maternal, cytoplasmic factors; expression of zygotic information begins at the blastula stage (Aoyagi *et al*., 1993; Nagler, 2000). Thus, in interspecific androgenesis, nuclear genomes follow cell cycles regulated in other species’ mode.

There are only few reports on interspecific diploid androgenesis. First successful attempt was performed on cyprinids (Bercsenyi *et al*., 1998), where androgenetic goldfish *Carassius auratus auratus* (L.) were produced in carp *Cyprinus carpio* L. eggs. Recoubratsky & Grunina (2001) obtained a few viable androgenetic carp larvae hatched out from hybrid goldfish × carp eggs. In salmonids, androgenetic development of rainbow trout in Yellowstone cutthroat trout *Oncorhynchus clarki bouvieri* (Richardson) eggs has been recently reported (Brown & Thorgaard, 2002).

The present study consists of two experiments, each aiming at induction of interspecies (between species) androgenesis in salmonid fishes. In Experiment 1, parameters of gamma irradiation and pressure shock application were tested in order to induce intraspecies androgenetic development in brown trout *Salmo trutta* L., brook trout and rainbow trout. Also, androgenetic and control crosses were performed between those species in order to induce interspecific and intergeneric hybrid development. In Experiment 2, based on results from Experiment 1, the optimum time of application of pressure shock for brown and brook trout intraspecies androgenesis was determined. Androgenetic development of Atlantic salmon *Salmo salar* L. and Arctic charr *Salvelinus alpinus* (L.) in eggs of brown and brook trout, respectively, was also induced in order to examine whether interspecies but intrageneric development in those fishes was possible.
EXPERIMENTAL DESIGN

The experiments were conducted in 1998 (Experiment 1) and 1999 (Experiment 2). Experiment 1 was performed on brown, brook and rainbow trout gametes. It aimed at optimization of intraspecies androgenesis in brown and brook trout and induced interspecies intergeneric androgenetic development between brown, brook and rainbow trout (Table I). Experiment 2 was performed on brown and brook trout oocytes and spermatozoa, and Atlantic salmon and Arctic charr spermatozoa. Based on results of Experiment 1, a further optimization of time of pressure shock application for intra-specific androgenesis in brown and brook trout was conducted. Interspecies, intrageneric (within genus) androgenetic development was also induced within two genera: Salmo and Salvelinus (Table I).

Optimization of intraspecies androgenesis

In Experiment 1, the effect of two processing factors was tested: gamma irradiation dose applied to destroy maternal nuclear genomes (35 or 50 kR) and the time of application of hydrostatic pressure shock (5 h 50 min or 7 h 30 min after insemination and incubation at 10°C). In variants where androgenesis was induced in oocytes of brown and brook trout, factorial design was full (two irradiation doses × two times of pressure application). Eggs of rainbow trout were irradiated only with a dose of 35 kR (Parsons & Thorgaard, 1985) but subjected to the pressure shock in the two previous settings: 5 h 50 min and 7 h 30 min. The settings of irradiation dose, were chosen following Babiak et al. (1998) who demonstrated that doses of 35 and 50 kR were equally efficient in rainbow trout, whereas doses of 65 kR or higher showed a lethal effect despite

*Irradiation dose 50 kR was not applied to OM oocytes.

MATERIALS AND METHODS

The experiments were conducted in 1998 (Experiment 1) and 1999 (Experiment 2). Experiment 1 was performed on brown, brook and rainbow trout gametes. It aimed at optimization of intraspecies androgenesis in brown and brook trout and induced interspecies intergeneric androgenetic development between brown, brook and rainbow trout (Table I). Experiment 2 was performed on brown and brook trout oocytes and spermatozoa, and Atlantic salmon and Arctic charr spermatozoa. Based on results of Experiment 1, a further optimization of time of pressure shock application for intraspecies androgenesis in brown and brook trout was conducted. Interspecies, intrageneric (within genus) androgenetic development was also induced within two genera: Salmo and Salvelinus (Table I).

Optimization of intraspecies androgenesis

In Experiment 1, the effect of two processing factors was tested: gamma irradiation dose applied to destroy maternal nuclear genomes (35 or 50 kR) and the time of application of hydrostatic pressure shock (5 h 50 min or 7 h 30 min after insemination and incubation at 10°C). In variants where androgenesis was induced in oocytes of brown and brook trout, factorial design was full (two irradiation doses × two times of pressure application). Eggs of rainbow trout were irradiated only with a dose of 35 kR (Parsons & Thorgaard, 1985) but subjected to the pressure shock in the two previous settings: 5 h 50 min and 7 h 30 min. The settings of irradiation dose, were chosen following Babiak et al. (1998) who demonstrated that doses of 35 and 50 kR were equally efficient in rainbow trout, whereas doses of 65 kR or higher showed a lethal effect despite
a high percentage of eyed embryos in haploid controls. The settings for time of application of pressure treatment, 5 h 50 min or 7 h 30 min after insemination, were chosen because they were optimal for rainbow and brook trout, respectively (Chourrout, 1984; Parsons & Thorgaard, 1985; May et al., 1988), and no data were available on brown trout. As the paternal effect on the rate of first mitotic division in the interspecific androgenetic zygote could not be excluded, those two settings were applied for each species, including rainbow trout.

In Experiment 2, eggs were subjected to the pressure treatment in 0·5 h intervals within 5–10 h (brown trout) or 5–8 h 30 min (brook trout) after insemination at 10° C.

**Induction of interspecies androgenesis**

In Experiment 1, individuals from the three species were crossed in reciprocal combinations. Also, androgenetic development was induced using spermatozoa and oocytes from each species in all possible combinations (three species × three species=9 combinations). In Experiment 2, crosses and induced androgenesis were conducted within two genera: *Salmo* (oocytes of brown trout and spermatozoa from brown trout or Atlantic salmon) and *Salvelinus* (oocytes of brook trout and spermatozoa from brook trout or Arctic charr).

**BREEDERS AND GAMETE COLLECTION**

In Experiment 1, gametes were collected in November 1998 from broodstocks of brown, brook and rainbow trout kept at the Department of Salmonid Research, Inland Fisheries Institute, Rutki, Poland. Oocytes were pooled within species from three 3 to 4 year old females. Spermatozoa were obtained from several 2 to 3 year old males and a single sample from each species was chosen for experiment after examination of spermatozoal motility.

In Experiment 2, gametes were collected from brown trout and Atlantic salmon (November 1999), and brook trout and Arctic charr (December 1999). Brown and brook trout gametes were obtained from broodstock cultivated in Rutki, Poland. Atlantic salmon semen was collected from wild spawners captured and kept in the Fishery Station at Swibno, Poland. Arctic charr semen was collected from 5 year old donors cultivated in the Saimaa Fisheries Research and Aquaculture Institute, Enonkoski, Finland. Oocytes were pooled within species from four brown trout females 4 years old and 14 brook trout females 2 years old. Sperm from brown trout, Atlantic salmon and brook trout was collected on the day of experiment. Arctic charr spermatozoa were obtained 9 days prior to the experiment and stored under oxygen at 0–1° C in Ziploc bags until used. A single sample from each species was chosen for experiment after examination of motility.

**TREATMENT**

After pooling, oocytes were kept submerged in coelomic fluid on crushed ice (0–2° C) until treated. Part of the oocytes were left intact to serve as controls, the remaining oocytes were subjected to a gamma irradiation as described by Babiak et al. (2002). Doses of either 35 kR or, excluding rainbow trout, 50 kR were used in Experiment 1, and a dose of 35 kR was applied in Experiment 2. Both control and irradiated batches contained c. 700 eggs per variant. They were inseminated with 0·08 ml of semen. Prior to insemination, 10 ml of fertilization diluent (154 mm NaCl and 1 mm Ca²⁺, buffered to pH 9·0 with 20 mm Tris+30 mm glycine; Billard, 1992) were added to each batch. Inseminated eggs were incubated at 10° C. To induce duplication of chromosome sets during the first mitotic division, batches of irradiated and inseminated batch were subjected for 4 min to 48 263 kN m⁻² hydrostatic pressure shock (Chourrout, 1984). This pressure shock was chosen because it proved to be optimal for rainbow trout (Babiak et al., 2002). In Experiment 1, the pressure shock was used at 5 h 50 min or 7 h 30 min after insemination. In Experiment 2, the pressure was applied every 30 min within 5–10 h (brown trout eggs) or 5 h–8 h 30 min (brook trout eggs) after insemination to
determine the optimal time of interruption of the first mitotic division. Control haploid batches of irradiated but not pressure-shocked eggs were performed for all experimental combinations. Experimental as well as control variants were incubated separately in three replicates at 7–9°C. Embryos were counted at the mid- to late eyed-egg stage, when embryo movements inside a chorion could be visible. All live embryos, including those showing morphological ‘aneuploid syndrome’, were counted. The progenies were counted again c. 2 weeks after hatching, at the mid yolk-sac resorption stage (c. 50% of the yolk sac was resorbed). All living larvae were classified as ‘hatched’ at this stage. The date of counting was corrected for differences in development rates of studied species.

MICROSATELLITE ANALYSIS

Microsatellite loci STR 73 (Estoup et al., 1993) and 85 (Estoup et al., 1998) were used for genetic identification of parental, hybrid and androgenetic genotypes in Salmo and Salvelinus, respectively. These loci showed interspecific polymorphism and no intraspecific polymorphism. Amplification was based on the primers described by Estoup et al. (1993, 1998). Genomic DNA was extracted from fin clips (parents) and from body pieces of eyed embryos (progeny) using a WIZARD® purification Kit (Promega, Madison, U.S.A.). Double stranded PCR amplifications were performed as described by Babiak et al. (2002). The amplified products were separated on 2% agarose gel (Promega, Madison, U.S.A.). The gels were stained with ethidium bromide (10 mg ml⁻¹) and photographed under UV light.

Microsatellite analyses were performed on: seven brown trout female donors; 14 brook trout female donors; four Atlantic salmon males; five Arctic charr males; 15 hybrid brown trout × Atlantic salmon progeny; 12 presumed interspecies androgenetic Atlantic salmon progeny; 15 hybrid brook trout × Arctic charr progeny; and 16 presumed interspecies androgenetic Arctic charr progeny.

STATISTICAL ANALYSIS

The data were organized, transformed and analysed according to Babiak (1998). The percentage values were transformed to follow a normal distribution, thus enabling the application of parametric procedures. The square root transformation was used ( \( T = \sqrt{P} \), where \( P \) is proportion of eyed eggs) for hatching rates in androgenetic groups, and arcsine square root transformation ( \( T = \text{arc sin} \sqrt{P} \) ) was used for percentages of eyed embryos (Anderson & McLean, 1974). The transformed data showed normal distribution, and the variances were homogenous (Cochran & Bartlett’s test). Multivariate analysis of variance (MANOVA) was used to determine the effect of tested factors and their interactions on the survival rates. A post hoc multiple range analysis (Duncan’s test) was used to estimate the significance of differences among the groups. Pearson’s product moment correlation coefficient was used to assess correlations.

RESULTS

INTRASPECIES ANDROGENESIS

Haploid control individuals did not hatch in any of the species.

In Experiment 1, the highest survival of androgenetic eyed embryos was 42·7, 25·1, and 20·5% in brown, brook, and rainbow trout, respectively. Androgenetic brown and brook trout hatched larvae were produced under each setting of irradiation dose and application of pressure shock (Fig. 1). Viable androgenetic rainbow trout larvae were obtained only when pressure shock was applied 5 h 50 min after insemination (mean ± s.d., 4·5 ± 1·4% of larvae at the mid yolk-sac resorption stage).
Analysis of variance for brown trout hatched larvae revealed that the application of pressure treatment at 7 h 30 min after fertilization was significantly superior to 5 h 50 min ($F_{1,8}=17.6$, $P=0.003$). The effect of irradiation dose was insignificant ($P=0.15$), similarly to the interaction between the irradiation dose and the time of application of pressure shock ($P=0.67$). The highest androgenetic yield in brown trout (mean ± s.d., 2.5 ± 0.9%) was obtained

![Graph of survival rates](image-url)
when oocytes were irradiated with 35 kR and were then treated with pressure shock 7 h 30 min after insemination [Fig. 1(a)].

Neither irradiation dose nor the time of application of pressure shock significantly affected androgenesis efficiency in brook trout \( (P=0.31 \text{ and } 0.11, \text{ respectively}) \). Also, the interaction between these two factors was insignificant \( (P=0.71) \). The highest survival rate of brook trout androgenetic larvae \( (4.3 \pm 1.9\%) \), not differing significantly from others, was obtained when 35 kR irradiated eggs were treated with pressure shock 5 h 50 min after insemination [Fig. 1(b)].

In Experiment 2, the highest survival of androgenetic eyed embryos of brown and brook trout was \( 20.5 \pm 6.7 \text{ and } 25.9 \pm 1.5\% \), respectively (Fig. 2). Androgenetic brown trout showed very poor survival at hatching. Few hatched larvae were recorded in variants where the pressure shock was applied at 7 h 30 min or later after insemination [Fig. 2(a)]. Due to low number of survivors, no statistical analysis was possible. The highest survival of androgenetic brook trout \( (3.3 \pm 0.8\%) \), not differing significantly from the others, was obtained in the variant with the application of pressure shock at 7 h after insemination. In this variant, the eyed-egg stage rate was also the highest [Fig. 2(b)]. The survival of androgenetic brook trout was significantly affected by the time of application of pressure shock \( (F_{7,16}=12.6, \text{ } P<0.0001) \). A post-hoc Duncan multiple range test revealed no significant differences among the time of application of pressure treatment in the range 5 h–7 h 30 min [Fig. 2(b)]. A very high correlation between survivals of brook trout androgenetic eyed embryos and hatched larvae \( (r=0.93, \text{ } P<0.05) \) was found.

**INTERSPECIES ANDROGENESIS**

In Experiment 1, where brown, brook and rainbow trout interspecies androgenetic development was induced in all reciprocal combinations, four of six hybrid crosses resulted in viable progeny (Table II). Androgenetic individuals were successfully produced, however, in only intraspecies variants. No developing embryos at the eyed-egg stage were observed in the interspecies androgenetic groups. The only interspecies androgenetic haploid controls viable at the eyed-egg stage \( (2.4 \pm 0.9 \text{ and } 1.3 \pm 2.3\%) \) were obtained after insemination of brown trout oocytes (irradiated with 35 and 50 kR, respectively) with brook trout spermatozoa. They did not hatch, however.

In Experiment 2, contrary to the results of Experiment 1, survival of interspecific androgenetic embryos was observed at the eyed stage (Fig. 2). The highest percentage of androgenetic Atlantic salmon developing in brown trout eggs was \( 12.7 \pm 3.2\% \) [Fig. 2(a)], whereas androgenetic Arctic charr developing in the brook trout eggs yielded \( 14.8 \pm 3.5\% \) of eyed embryos in the best variant [Fig. 2(b)]. Despite this, and the high survival of control hybrid larvae (Table II), none of interspecific androgenetic individuals survived until hatching.

The highest survival at the eyed-egg stage of both interspecies Atlantic salmon and intraspecies brown trout androgenetic embryos occurred when pressure shock was applied 9 h after insemination [Fig. 2(a)]. These results, however, do not differ statistically from the others. A very high correlation \( (r=0.89, \text{ } P<0.05) \)
between survival in these two species at the stage of eyed embryos was determined. Androgenetic Arctic charr eyed embryos, similarly to brook trout, showed the highest survival when the pressure shock was applied 7 h after insemination [Fig. 2(b)]. A very high correlation between survival of brook trout and Arctic charr androgenetic eyed embryos \((r=0.93, P<0.05)\) was found.

**Fig. 2.** Experiment 2: The effect of time of pressure treatment application on survival at the eyed embryos and hatching stage of intra-and interspecific androgenetic (a) *Salmo trutta* and *Salmo salar*, produced in eggs of *S. trutta*, and (b) *Salvelinus fontinalis* and *Salvelinus alpinus*, produced in eggs of *S. fontinalis*. None of interspecific androgenetic individuals survived hatching. Within stages (eyed-egg or hatching) and species, values marked with the same letter do not differ significantly from each other (Duncan’s multiple range tests, \(P<0.05\)). (a) S. trutta hatched larvae; ◆, S. trutta eyed embryos; ■, S. salar eyed embryos. (b) S. fontinalis hatched larvae; ◆, S. fontinalis eyed embryos; ■, S. alpinus eyed embryos.
MOLECULAR EXAMINATION OF ANDROGENETIC INDIVIDUALS

Microsatellite primers, both STR 73 and 85, showed species-specific polymorphism and consistent lack of intraspecies polymorphism. Two alleles at locus STR 73 in samples from the genus Salmo were detected. Homozygous genotypes were revealed for Atlantic salmon male and brown trout females, AA (180/180 bp) and BB (160/160 bp), respectively [Fig. 3(a)]. All control hybrid progeny possessed a presumed genotype AB (180/160 bp). Interspecific androgenetic progeny showed either a paternal genotype AA or hybrid genotype AB; the latter indicated the presence of maternal nuclear DNA in the examined specimens.

Three allelic forms were revealed at locus 85 within the Salvelinus genus [Fig. 3(b)]. The Arctic charr paternal genotype was heterozygous AB (A=145 bp and B=130 bp), whereas maternal genotypes were homozygous CC (120 bp). Control hybrid progeny possessed one of two expected genotypes (AC or BC). Interspecific androgenetic progeny exhibited only paternal genotypes; individuals were homozygous for either allele A or B (frequencies 0·5:0·5, n=16)

DISCUSSION

INTRASPECIES ANDROGENESIS

This is the first report on induced androgenesis in the genus Salmo. The survival of 42·7% of brown trout eyed embryos in the best variant of Experiment 1 [Fig. 1(a)] is one of the highest reported on induced androgenesis in fishes (Ihssen et al., 1990; Pandian & Koteeswaran, 1998), comparable to the results for
rainbow trout (Babiak et al., 2002). Despite this high survival of brown trout eyed embryos, only 2.5% larvae hatched, which is ten times lower than for rainbow trout (Babiak et al., 2002). As it was revealed in Experiment 2, optimal time of application would be c. 9 h after insemination, thus a pressure shock applied earlier probably led to an increase of the aneuploidy level in treated

![Diagram showing genotypes for the microsatellite locus](image)

**Fig. 3.** Experiment 2: (a) interspecific androgenetic development of *Salmo salar* in eggs of *Salmo trutta*: genotypes for the microsatellite locus STR 73 (Estoup et al., 1993). Maternal BB and paternal AA genotypes are presented in lanes 1 and 9, respectively, and their hybrid progeny in lane 2 (AB). Androgenetic progeny exhibited two genotypes: heterozygous AB (lanes 3, 4 and 5) and homozygous AA (lanes 6, 7 and 8). Lane 10: pUC 18 DNA/Hae III DNA ladder; (b) interspecific androgenetic development of *Salvelinus alpinus* in eggs of *Salvelinus fontinalis*: genotypes for the microsatellite locus 85 (Estoup et al., 1998). Maternal CC and paternal AB genotypes are presented in lanes 1 and 7, respectively, and their hybrid progeny in lane 2 (BC). Androgenetic progeny showed either AA (lane 5) or BB (lanes 3, 4 and 6) genotypes. Lane 8: ϕX 174 DNA/Hinf I DNA ladder. Schematic representation of genotypes: maternal, ○; paternal, ◦; hybrid, ★.
embryos in Experiment 1. Unfortunately, the poor quality of oocytes in Experiment 2 did not allow for the improvement of androgenesis efficiency. As none of androgenetic brown trout survived for more than 4 months, further optimization of the protocol is needed.

Hatching of androgenetic brook trout has only been reported to date by May et al. (1988), however, the authors did not present data on hatching rates or further survival. In the present study, hatching rates were relatively low despite high percentages of eyed embryos [Figs 1(b) and 2(b)]. Nevertheless, a few androgenetic individuals survived 2 years. The results of Experiment 2 indicate that optimal time for application of pressure shock was 7 h after insemination and incubation at 10°C [Fig. 2(b)]. May et al. (1988) found 7 h 30 min to be optimal. This 30 min difference might be because the rate of the first mitotic division varies among different strains (Scheerer et al., 1991). The protocol used in the present study proved its suitability for production of androgenetic brook trout.

Results obtained with androgenetic rainbow trout (4.5% of mid-yolk-sac resorption larvae), although comparable with most of others in this species (Pandian & Koteeswaran, 1998), are rather low when compared to Babiak et al. (2002). The reason probably lies in the genetic origin of oocyte donors, a critical biological factor in induced androgenesis (Babiak et al., 2002). Fish used in the present experiment originated from a strain not selected for egg quality, contrary to the strain used by Babiak et al. (2002).

INTERSPECIES HYBRIDIZATION AND ANDROGENESIS

Hatching rates of control intergeneric hybrids in Experiment 1 exceeded 60% in crosses ST × SF, SF × ST and OM × SF, while hatching of hybrids OM × ST was poor, and no viable larvae were obtained in crosses ST × OM and SF × OM. In Experiment 2, hatching rates of intrageneric hybrids ST × SS and SF × SA were relatively high (Table II). These data are in agreement with results reported by other authors (McKay et al., 1992; Gray et al., 1993). High mortality was observed in all hatched hybrids and until they reached alevin stage (unpubl. data). Contrary to the pre-hatch mortality in inviable crosses, which is caused by the chromosome elimination and resulting lethal aneuploidy (Fujiiwara et al., 1997), the reasons of the post-hatch mortality are unclear. Incomplete karyogamy, caused by different numbers of maternal and paternal chromosomes, would lead to aneuploidy (Chevassus, 1983), probably by chromosome elimination. If such an event occurs in intergeneric hybrids, which are functionally sterile (Chevassus, 1983), this does not apply to intrageneric ST × SA and SF × SA hybrids, which can be fertile (Johnson & Wright, 1986; Johnson et al., 1987) and possess intermediate number of paternal chromosomes (Disney & Wright, 1987).

Interspecific androgenesis was successfully induced in cyprinids (Bercsenyi et al., 1998; Recoubratsky & Grunina, 2001) and in Oncorhynchus sp. (Brown & Thorgaard, 2002). The present study demonstrates that this does not directly apply to other salmonids. No viable interspecific androgenetic individuals were obtained from recipient eggs originating from other genera (Experiment 1). Even using recipient oocytes from other species within the same genus (Experiment 2) resulted in no viable larvae. Genetic relationships between the
species used were close enough to produce viable hybrids in four of six possible crosses in Experiment 1 and in two of two crosses in Experiment 2. Moreover, androgenetic progeny hatched in all intraspecies variants, where oocytes and sperm originated from the same species. This indicates that lack of maternal nuclear DNA is a primary lethal factor preventing interspecific androgenesis in tested species and not hybrid development (except crosses with rainbow trout males) or the androgenesis process.

In Experiment 1 (intergeneric androgenesis), the development ceased earlier than in Experiment 2 (intrageneric androgenesis). The only intergeneric androgenetic eyed embryos (androgenetic brook trout produced in brown trout eggs) were obtained in haploid controls. A similar low survival of haploid androgenetic brook trout, developing in brown trout eggs, was observed by May & Grewe (1993). In Experiment 2, a relatively high percentage of eyed embryos was observed in interspecific androgenetic groups (Fig. 2). Optimal time of application of pressure treatment was the same for androgenetic brown trout and Atlantic salmon [9 h; Fig. 2(a)], as well as for brook trout and Arctic charr (7 h; Fig. 2(b)). Also, correlations between androgenetic eyed embryos rates in *Salmo* and *Salvelinus* were very high (0·89 and 0·93, respectively), indicating that the rate of the first cell division within these two pairs was approximately the same. Nevertheless, none of interspecific androgenetic larvae hatched.

Non-nucleated salmonid embryos can develop until the blastula stage (Aoyagi et al., 1993) and the nuclear genome is not active until blastulation (Nagler, 2000). Maternal cytoplasmic factors exclusively control early development in fishes until the midblastula transition, MDT (Kane & Kimmel, 1993). It has been demonstrated that regulatory signals controlling development of zebrafish *Brachydanio rerio* (Hamilton-Buchanan) embryos are exclusively maternally-derived until MDT (Shimizu et al., 2000; Braat et al., 2000; Gore & Sampath, 2002). Even when zygotic information is expressed, a number of cytoplasmic signals is involved in modulation of a nuclear genome in mammals (Cummins, 2001; Fulka et al., 2001). In the case of interspecies nucleo-cytoplasmic hybrids, this regulatory incompatibility leads to chromosome elimination during early development; demonstrated by Fujiwara et al. (1997) on inviable masu salmon *Oncorhynchus masou* (Brevoort) × rainbow trout hybrids. These authors revealed that the elimination of chromosomes was uniparental in the case of nuclear hybrids, with preference to eliminate paternal chromosomes, yet still occurred in the absence of the maternal masu salmon genome in haploid androgenetic rainbow trout embryos. This effect could result in ceasing the development of interspecific androgenetic embryos in the present study. Karyotypical and developmental examination of embryos at early stages would help to understand the reasons of inviability of interspecies androgenotes.

Failure of interspecies androgenesis in the present study stands in contrast to successful attempts reported in cyprinids and *Oncorhynchus* sp. Rainbow trout and Yellowstone cutthroat trout, used by Brown & Thorgaard (2002), show similar karyotypical characteristics (Hartley, 1987) and their hybrids are fully fertile and developmentally compatible (Ferguson et al., 1985) as are cyprinids (Kirpichnikov, 1981; Liu et al., 2001). Species used in the present study, even within the same genus, karyolotypically differ much more (Hartley, 1987) and their hybrids show poor fertility if any (Chevassus, 1983; Galbreath &
These data suggest that interspecific androgenesis is possible only in very closely related species, thus different genera of cyprinids are in fact genetically more closely related than salmonid species within the genera *Salmo* and *Salvelinus*.

Phylogenetic relationships among Salmonidae are complicated and, despite extensive studies, no satisfying, unequivocal model has been defined to date (Oakley & Phillips, 1999; Osinov & Lebedev, 2000). DNA-based research on phylogeny in Salmonine has led Oakley & Phillips (1999) to the conclusion that *Oncorhynchus* and *Salmo* are not sister genera, contrary to the commonly accepted hypothesis. One possible phylogenetic model within Salmonine, proposed by Oakley & Phillips (1999), assumes a closer relationship between *Salmo* and *Salvelinus* than between *Oncorhynchus* and these two genera. The present reproductive data seem to support this model, as reciprocal hybrids of brown and brook trout were viable, contrary to hybrids of these two species with rainbow trout males (Table II), and the only interspecific development to the eyed-egg stage was observed in haploid controls of brook trout developing in brown trout eggs.

**MOLECULAR EXAMINATION OF THE PROGENY**

All examined androgenetic Arctic charr eyed embryos showed homozygous Arctic charr paternal phenotypes [Fig. 3(b)], whereas the part of the presumably androgenetic salmon contained maternal brown trout nuclear DNA [Fig. 3(a)]. This indicates an incomplete nuclear inactivation of brown trout oocytes. The need for further development of androgenesis protocols for brown trout focusing on irradiation dose seems to be obvious, however, implications are much broader. The genetic control in a single microsatellite locus, as in the present study, is sufficient for confirming homozygosity and paternal origin of resulting embryos, yet probably insufficient to exclude maternal DNA remains. Using more 'points of control' (more microsatellites, RAPD, AFLP) would increase the probability for detection of maternal contamination, but still not guaranteeing that no maternal DNA persists. The irradiation doses used in the present study were equal to or higher than doses considered as optimal for enucleation of rainbow trout oocytes (Scheerer et al., 1991). Brown trout oocytes are larger than those of rainbow trout, but an oocyte size should not be critical when using permeating irradiation, such as gamma rays. Therefore, the presumed lack of maternal nuclear DNA in every androgenetic individual produced using oocyte irradiation may be questionable.

The intriguing question is what happens with maternal DNA fragments after irradiation. Karyological examination of adult, 3 year old androgenetic rainbow trout revealed extra chromosome fragment-like structures in some individuals (K. Ocalewicz, S. Dobosz, K. Goryczko, H. Kuzminsinski & I. Babiak, unpubl. data). This observation and the present study do not exclude a possibility that maternal nuclear genome fragments can persist after numerous cell divisions, therefore, they can be replicated. Besides an extremely high inbred level (Scheerer et al., 1991), these unintended remains of maternal genome might be an additional reason for poor viability of androgenetic larvae, reported by Babiak et al. (2002). Maternal genomic DNA contamination may result also in high
mortality of eyed embryos, observed in the present study in both intra- and interspecies androgenetic groups, and reported by other authors (Pandian & Koteeswaran, 1998).

We are much indebted to T. Hartman for critical reading the manuscript. The study was supported by the Polish Committee for Scientific Research, Project 5 P06D 010 13.

References


