Transgene transmission in South American catfish (*Rhamdia quelen*) larvae by sperm-mediated gene transfer

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The silver catfish (*Rhamdia quelen*) is an endemic American fish species. The sperm of each species has its own peculiarities and biological characteristics, which influence the success of mass DNA transfer methods. Our objective in this study was to evaluate different sperm-mediated gene transfer (SMGT) methods to obtain transgenic silver catfish. Different treatments for the incorporation of a foreign pEGFP plasmid group were used: (1) dehydrated/rehydrated (DR), (2) dehydrated/rehydrated/electroporated (DRE), (3) electroporated (E), (4) incubated with seminal plasma (INC); and (5) incubated in the absence of seminal plasma (INCSP). Sperm motility, time of activity duration (TAD), fertilization rate (FR), hatching rate (HR) and sperm morphology were also evaluated. The polymerase chain reaction (PCR) positivity rates for the presence of the transgene were: DRE 60%; DR 40%; E 25%; INC 5% and INCSP 25%. The rates of embryo EGFP expression were: DRE 63%; DR 44%; E 34%; INC 8% and INCSP 38%. The fertilization rate in the control and DRE treatments groups were higher than in the DR group, but the E, INC and INCSP treatment groups had the lowest rate. The hatching rates of the DRE, DR and control groups were higher than in the INCSP, INC and E treatment groups ($P>0.05$). There were no differences among the DRE and DR, E and DR, E and INCSP groups in expression and PCR positivity rates of enhanced green fluorescent protein (EGFP) in embryos. Scanning electron microscopy also did not show any change in sperm morphology among treatment groups. To the best of our knowledge, this is the first report on transgene transmission of exogenous DNA into silver catfish larvae through SMGT technology.

[Collares T, Campos V F, Seixas F K, Cavalcanti P V, Dellagostin O A, Moreira H L M and Deschamps J C 2010 Transgene transmission in South American catfish (*Rhamdia quelen*) larvae by sperm-mediated gene transfer; *J Biosci.* **35** 39–47] **DOI** 10.1007/s12038-010-0006-6

1. Introduction

The silver catfish (*Rhamdia quelen*) is a teleost species from the Siluridae family and is an important species for aquaculture in temperate and subtropical climates. Silver catfish are found from southern Mexico to central Argentina, and husbandry of this species is spreading towards southern Brazil. Fish farmers are interested in culturing this species because of its growth rate, omnivorous feeding habit, high fertilization and hatching rates, and good acceptance by consumers (Barcellos *et al.* 2001*,* 2006; Reidel *et al.* 2010). The biological characteristics of semen ejaculates of this species are of interest in aquaculture biotechnology (Borges *et al.* 2005).

In the past, microinjection of plasmid DNA into early embryos represented the state of the art in generating transgenic fish. However, this approach suffers from significant drawbacks (mosaic distribution of the injected transgene, late transgene integration at high copy numbers, low transgenesis frequency), making the generation of

Keywords. Catfish; electroporation; osmotic differential; seminal plasma; SMGT; transgene

Abbreviations used: AS, activation solution; DR, dehydrated/rehydrated; DRE, dehydrated/rehydrated/electroporated; E, electroporated; EGFP, enhanced green fluorescent protein; FR, fertilization rate; GFP, green fluorescent protein; hCG, human chorionic gonadotrophin; HR, hatching rate; INC, incubated with seminal plasma; INCSP, incubated in the absence of seminal plasma; PCR, polymerase chain reaction; SM, sperm motility; SMGT, sperm-mediated gene transfer; TAD, time of activity duration

transgenic lines a laborious task (Soroldoni *et al.* 2009). Thus, it is necessary to develop simple gene transfer methods for use in aquaculture. During the past few years, spermatozoa have been studied to serve as a vector for gene transfer in transgenic animal technology and several different approaches have been developed. Several transgenic animals have been successfully produced by sperm-mediated gene transfer (SMGT) (Brackett *et al.* 1971; Lavitrano *et al.* 2002; Shen *et al.* 2006; Coward *et al.* 2007; Harel-Markowitz *et al.* 2009). However, few studies have reported on the use of sperm as vector of exogenous DNA for generation of transgenic fish (Khoo *et al.* 1992; Kang *et al.* 1999; Lu *et al.* 2002; Lanes *et al.* 2009). Spermatozoa of virtually all species can take up exogenous DNA or RNA molecules and internalize them into nuclei (Sciamanna *et al.* 2009). Naturally, aquatic animals produce a huge number of sperm cells, which is an advantage for SMGT techniques. On the other hand, each fish species has a particular reproductive biology necessitating the development of specific SMGT protocols.

Here, we describe the transmission of the enhanced green fluorescent protein (EGFP) transgene in silver catfish by sperm cells subjected to SMGT by comparing the osmotic differential and/or electroporation and incubation in the presence or absence of seminal plasma as a means of increasing the rate of transgenic embryos.

2. Materials and methods

2.1 *pEGFP-N1 vector preparation*

The pEGFP-N1 plasmid was purchased from CLONTECH (Mountain View, CA, USA). Its whole length was 4.7 kb. EGFP was expressed under the control of the CMV promoter. EGFP has a single excitation peak at 488 nm and a maximal emission peak at 507 nm. The plasmid was first amplified in *Escherichia coli* cells and subjected to large-scale DNA extraction and purification using the Perfectprep Plasmid Maxi kit (Eppendorf, Germany®). A proportion of 3:1 of linear:circular DNA was used as exogenous DNA to transfect silver catfish sperm cells at a total of 30 μg/ml according to Shen *et al.* 2006. The linearized vector was digested with *Apa* LI restriction enzyme.

2.2 *Collection and manipulation of gametes*

The experiments were conducted using six adult male silver catfish with an average body weight of 534 g and average total length of 38 cm. Animals were maintained at the UFPel Aquaculture Station, in 500 l fibre tanks with closed water circulation. Human chorionic gonadotrophin (hCG) at 400 UI/kg was used for inducing artificial spermiation. After 8 h of induction, semen was collected by immobilizing and blinding the animals with a humidified towel to avoid stress and injuries. The urogenital pore was dried with a paper towel to reduce the possibility of contamination with water, faeces or urine, and consequent sperm activation with NaCl (50 mOsm/kg). Semen was aspirated with graduated syringes (5.0 ml), stored in properly identified 15 ml *Falcon* tubes, and maintained at 8ºC during transportation to the Federal University of Pelotas Biotechnology Center, where the treatments were performed.

Immediately after collection, 5 μl of fresh semen from each fish was placed on slides and tested for sperm activation by fluid contamination using an optical phase-contrast microscope (200x). Considering that sperm in non-contaminated fresh fish semen are non-motile, samples that exhibited no sperm motility were considered adequate for the experiments.

To access female gametes and perform *in vitro* fertilization, three female silver catfish were induced with 800 UI of hCG/kg of live weight. The average corporal weight of the females used in this experiment was 1120 g and the average total length was 44 cm. After 12 h of hormonal induction, the eggs were collected in plastic buckets by *hand stripping*, in synchrony with the sperm manipulation treatments.

2.3 *Sperm diluents*

Three diluents with different osmolalities (isosmotic, hyperosmotic and hyposmotic) were used. The ejaculates collected from the 6 male donors were evaluated for sperm osmolarity using a Wescor 5500 vapour pressure osmometer before sperm dilution. The isosmotic solution was based on catfish sperm diluents, often used in fish sperm cryopreservation processes (Christensen and Tiersch 1997). These diluents represent osmolalities of approximately 300 mOsm/kg, similar to the average of the donor ejaculates obtained in previous trials (305 mOsm/kg). and do not allow sperm activation after dilution.

Semen samples were stored in 1.5 ml microtubes and incubated at 5ºC in three different catfish diluents: hyperosmotic (595 mOsm/kg), isosmotic (305 mOsm/kg), and hyposmotic (125 mOsm/kg).

The sperm samples were submitted to five treatments: treatment 1 – dehydration and rehydration (DR), treatment 2 – dehydration, rehydration and electroporation (DRE), treatment 3 – only electroporation (E), treatment 4 – incubation with seminal plasma (INC); and treatment 5 – incubation in the absence of seminal plasma (INCSP). The control consisted of fresh semen samples diluted in isosmotic media without exogenous DNA. Dehydrated sperm was obtained by incubation in hyperosmotic (595 mOsm/kg) catfish diluents for 1 h at 5ºC. For rehydration, the dehydrated sperm was mixed with an equal volume of

hyposmotic (125 mOsm/kg) catfish diluents containing foreign DNA, yielding a medium that was isosmotic to the original seminal fluid at about 320 mOsm/kg. DR sperm samples were submitted to electroporation. This was conducted with 3.0 V capacitance, 200 ohms and 2.5 kV for all electroporated samples using a MicroPulser Electroporator (Bio-rad®, USA). INC treatment was done by incubation in a sperm:isosmotic medium DNA complex at 1:1 ratio for 1 h at 5ºC. For the INCSP treatment, the samples were washed by centrifugation (500 × *g* for 10 min at 5ºC) to remove the seminal plasma. The pellet was quickly and gently dissolved in isosmotic medium DNA complex. There was no sperm activation of the sperm cells during the dilutions. For sperm activation, NaCl (50 mOsm/kg) activation solution (AS) was used in a semen:AS proportion of 1:10, allowing analyses of sperm motility and time of activity duration (TAD), both performed in an optical phase-contrast microscope (200x).

2.4 *Sperm morphology*

Due to the reduced dimensions of the silver catfish sperm observed on optical microscopy, sperm morphology of the proposed treatments was visualized by scanning electron microscopy to evaluate sperm integrity and determine the cellular dimensions of the spermatozoa of this species. Semen was dropped onto cover glasses and fixed in 2.5% glutaraldehyde in water. After they were post-fixed in 1% osium tetroxide, dehydrated with graded ethanol and dried by the critical point method, they were observed under a JEOL JOM 7000 scanning electron microscope.

2.5 In vitro *fertilization*

After treatment, semen samples were transported from the UFPel Biotechnology Center to the UFPel Aquaculture Station for *in vitro* fertilization to be performed under controlled conditions. At the station, sperm motility (SM) and TAD analyses after treatment and transport were measured.

Previously induced females were extruded for collection of eggs. These were weighed and split onto glass plates for fertilization with the sperm from each treatment. Approximately 9000 eggs were used in each treatment (3000 eggs per repetition), totalling 54 000 eggs. Three thousand eggs were mixed with 1 ml of treated semen, stirred with a glass stick and then activated with 150 ml of activation solution (NaCl 50 mOsm/kg). After fertilization, eggs were maintained in submerged incubators, in pools with water properly controlled for embryo development at standard temperature (23ºC), pH (7.6), CO_2 (0.5%), alkalinity (21%) and oxygen saturation (75%).

2.6 *Evaluation of EGFP expression*

Hatched larvae were maintained in properly controlled pools for 96 h post-hatching. After 100 h of embryo development, the animals, measuring approximately 0.8 cm, were evaluated for green fluorescent protein (GFP) gene expression under a fluorescence microscope. Ninety animals per treatment were evaluated. All animals that presented any variable degree of expression during fluorescence analysis were considered positive for EGFP.

2.7 *PCR and sequencing analyses*

Twenty of the 90-day-old animals from each treatment were randomly selected for genomic DNA extraction from muscle tissue. DNA was obtained by the PureLink™ Genomic DNA Purification Kit (Invitrogen®, USA). Polymerase chain reaction (PCR) was performed with pEGFP-N1-specific oligonucleotides (5′- CGGGACTTTCCAAAATGTCG -3′ and 5′- GAAGATGGTGCGCTCCTGGA -3′) to amplify a 500 base pair (bp) fragment. PCR reactions were carried out in an Mastercycler gradient thermal cycler (Eppendorf®, Germany) programmed for an initial denaturation step (2 min at 94°C) followed by 30 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C. The last cycle was followed by a final incubation of 7 min at 72°C. The samples were then stored at –20°C until use. Each PCR product was sequenced in the automatic sequencer MegaBace 500 (Amersham Biosciences).

2.8 *Statistical analyses*

ANOVA was used to evaluate the effect of the treatments on SM, TAD, fertilization rate (FR), and hatching rate (HR), followed by the Duncan test for means comparison (SAS Institute Inc., Cary, NC). Fisher exact tests and Pearson chi-square were used to evaluate EGFP expression and PCR positivity rates. Differences were considered to be statistically significant at the 95% confidence level (P<0.05). Pearson correlation coefficients among variables were tested.

3. Results

3.1 In vitro *reproductive parameters*

Statistical analyses demonstrated that the treatments had a significant effect (P<0.05) on motility, TAD, FR and HR (table 1).

3.2 *Sperm motility*

DRE treatment resulted in motility parameters higher than that with the control treatment (P<0.05). This was

probably due to a reflex of the osmotic differential plus the electroporation that promoted a reorganization of the plasmatic membrane with activation of the sodium and potassium pumps, stimulating sperm motility and cell activity. Motility in the DR treatment group did not differ from that in the control group, but differed when compared with the other groups. The electroporation group demonstrated significant loss of motility compared with the control, DR and DRE groups; however, there was no difference when compared with the incubation treatments group in the presence or absence of seminal plasma and foreign DNA. We observed that removal of the proteins of seminal plasma, as well as other seminal constituents, promoted a significant loss of motility in the INCSP group ($P<0.05$). Based on the Pearson correlation coefficients, it was possible to observe a significant correlation between motility x TDA and motility x FR, but not between motility and HR. This demonstrates that other factors besides the presence of foreign DNA can influence the HR.

3.3 *Time of activity duration (TAD)*

The TAD, in other words, the duration for which the spermatozoa remain motile, is important because it is the length of time that the spermatozoa interact with female gametes during the fertilization process. Statistical analyses for this parameter separated the treatments in five significantly different groups (table 1). The TAD for the control group (217.0±9.0 s) was higher than that for the groups that underwent the other treatments ($P<0.05$). There was no difference in the TAD between the DR and DRE treatment groups. The TAD following E treatment was significantly higher when compared with the INC and INCSP treatments; however, it was significantly lower than the DR, DRE and control treatments. A low TAD rate (44.6±5.5 s) was observed in the INCSP treatment group. Removal of the constituents of seminal plasma results in the loss of an energy source to maintain sperm motility. It was possible to observe a significant correlation between TAD × motility, TAD × FR and TAD × HR (table 2).

3.4 In vitro *fertilization rate (FR)*

Statistical analyses for the FR parameter separated the treatments into three significantly different groups (table 1). The FR following DRE treatment (90.3±0.58%) did not differ from that of the control (89.0±5.29%) but differed from that of the other treatments. DR treatment resulted in a lower FR (78.0±2.65%) than DRE and control treatments but was significantly higher when compared with the other treatments, which did not differ among themselves (E=64.6±5.03%; INC=65.3±4.16% and INC SP=69.6±0.58%). Table 2 shows a significant correlation between FR and motility. However, the FR in the control treatment group did not differ from that of the DRE treatment group although motility rates were significantly different ($P<0.05$). This effect might be due to cellular exhaustion resulting from the increase in motility due to the osmotic

Table 1. Averages of the treatments for the analysed variables

Treatments	Motility (%)	TAD (s)	IVF (%)	HR (%)	GFP (%)	PCR (%)
Control	76.6±5.77 ^{b}	217.0±9.00 ^{a}	89.0±5.29 ^{a}	90.0±0 ^{a}	0 (0/90) ^{d}	0 (0/20) ^{c}
DR	80.0±0 ^{b}	179.6±17.50 ^{b}	78.0±2.65 ^{b}	90.0±0 ^{a}	44 (40/90) ^{ab}	40 (8/20) ^{ab}
DRE	90.0±0 ^{a}	171.3±8.08 ^{b}	90.3±0.58 ^{a}	88.3±2.89 ^{a}	63 (57/90) ^{a}	60 (12/20) ^{a}
E	56.6±5.77 ^{cd}	91.3±7.09 ^{c}	64.6±5.03 ^{c}	73.3±5.77 ^{b}	34 (31/90) ^{b}	25 (5/20) ^{b}
INC	53.2±5.77 ^{d}	65.0±5.00 ^{d}	65.3±4.16 ^{c}	73.3±2.89 ^{b}	8 (7/90) ^{c}	5 (1/20) ^{c}
INC SP	63.2±5.77 ^{c}	44.6±5.51 ^{e}	69.6±0.58 ^{c}	71.6±2.89 ^{b}	38 (34/90) ^{b}	25 (5/20) ^{b}

Treatments: DR, dehydrated/rehydrated; DRE, dehydrated/rehydrated/electroporated; E, electroporated; INC, sperm incubation with seminal plasma; INCSP, sperm incubation in the absence of seminal plasma. Parameters: sperm motility; TAD, time of activity duration; IVF, *in vitro* fertilization rate; HR, hatch rate; GFP, embryo expression of green fluorescent protein (GFP); PCR, PCR analyses.

^{a,b,c,d,e} differ significantly ($P<0.05$) in the same column.

Table 2. Pearson correlation coefficients among tested variables

	Motility	HR	IVF
TAD	0.7994*	0.8507*	0.8720*
IVF	0.9196**	0.5984 ns	—
HR	0.6202 ns	—	—

ns, did not differ significantly; * $P<0.05$; ** $P<0.01$ Parameters: sperm motility; TAD, time of activity duration; IVF, *in vitro* fertilization rate; HR, hatch rate;

differential plus the electroporation, which can be observed in the TAD of the DRE and control groups..

3.5 *Hatching rate (HR)*

The embryo HR is the percentage of embryos that emerge from fertilized eggs. For this parameter, two groups with a significant statistical difference were observed. The control, DR and DRE treatment groups did not differ among themselves but they differed from the E, INC and INCSP treatment groups, which again did not differ among themselves (table 1). Hatching rates increased when an osmotic differential was applied to sperm cells, showing that electroporation is not necessary to increase this rate. Incubation of sperm cell/DNA, in the presence or absence of seminal plasma in isosmotic conditions, demonstrates the same effect for this parameter. Pearson correlation coefficients among tested variables demonstrated that the HR had a significant correlation only with the TAD (r=0.85), indicating that the duration of the sperm activity can determine the success of the HR of eggs, regardless of the presence of foreign DNA (table 2). The low correlation between FR and HR (r=0.59) can be a result of the interference in gene regulation of embryonic development caused by the foreign DNA or the effect of manipulation of the gametes.

3.6 *Sperm morphology*

Morphology data, not previously published, which characterize the morphology of *Rhamdia quelen* spermatozoa, were measured: head with a diameter of 1 μm and length of 2 μm; tail with diameter of 120 nm and length of 20 μm. The presence of an acrosome was not observed in these cells. Sperm morphology demonstrated agglutination of cells with the E and INCSP treatments, which was not observed with the other treatments (figure 1), but there was no loss of integrity with all the treatments.

Sperm pathology was observed in 20% of all samples analysed, which was acceptable. This was the result of procedures done for sperm manipulation and injury caused by the treatments. However, this did not affect the fertilization process of each treatment.

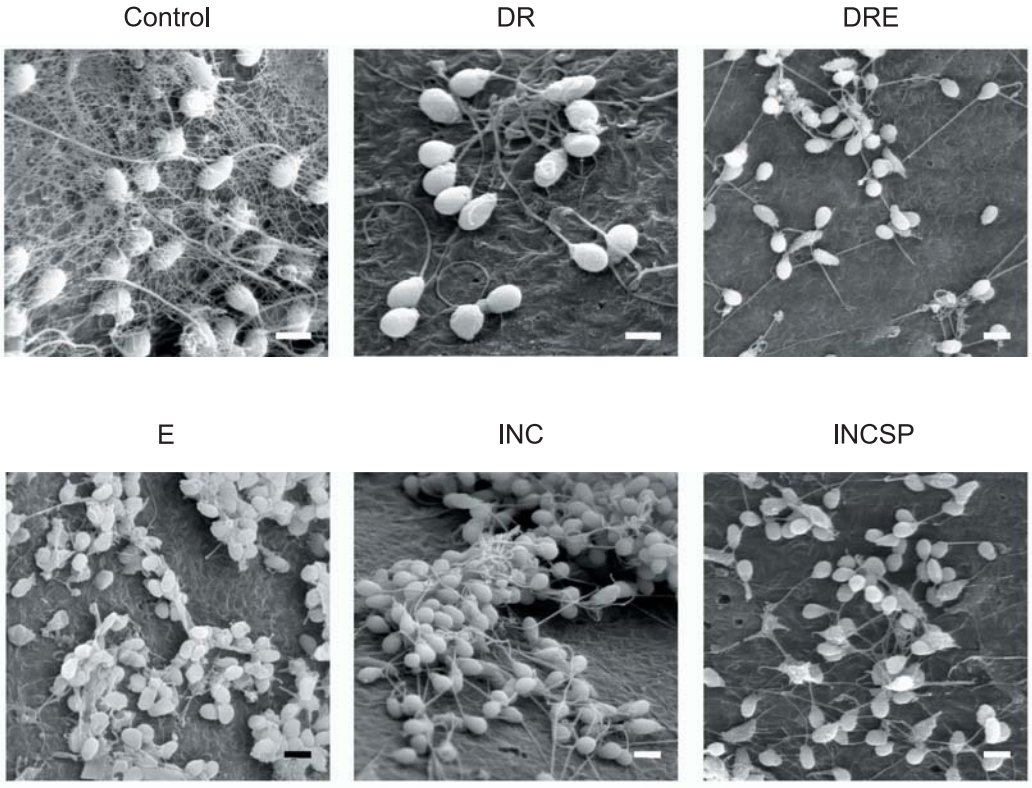

Figure 1. Scanning electron microscopy of silver catfish spermatozoa after treatments: DR, dehydrated/rehydrated; DRE, dehydrated/rehydrated/electroporated; E, electroporated; INC, sperm incubation with seminal plasma; INCSP, sperm incubation in the absence of seminal plasma. Scale bars = 2 μm.

3.7 *Transient EGFP expression and PCR analyses*

All the treatments were capable of generating animals with different degrees of transient GFP expression in muscle and/or nervous tissue, except for the control group (figure 2). A total of 44% (40/90) and 63% (57/90) of transgenic fish from the DR and DRE treatment groups were observed, respectively. These groups did not differ significantly ($P>0.05$), demonstrating that E did not increase the expression rate. In addition, the results of DR treatment did not differ from those of E treatment (34%, 31/90), indicating that only osmotic differential can promote the uptake of foreign DNA and determine the same expression rate; consequently, it is not necessary to use electroporation equipment. INCSP treatment resulted in an expression rate (38%, 34/90) that did not differ from that with E treatment, demonstrating that removal of the biological constituents of seminal plasma can lead to an increase in the incorporation of foreign DNA without the need for sophisticated equipment. INC treatment resulted in a lower expression rate, with 8%

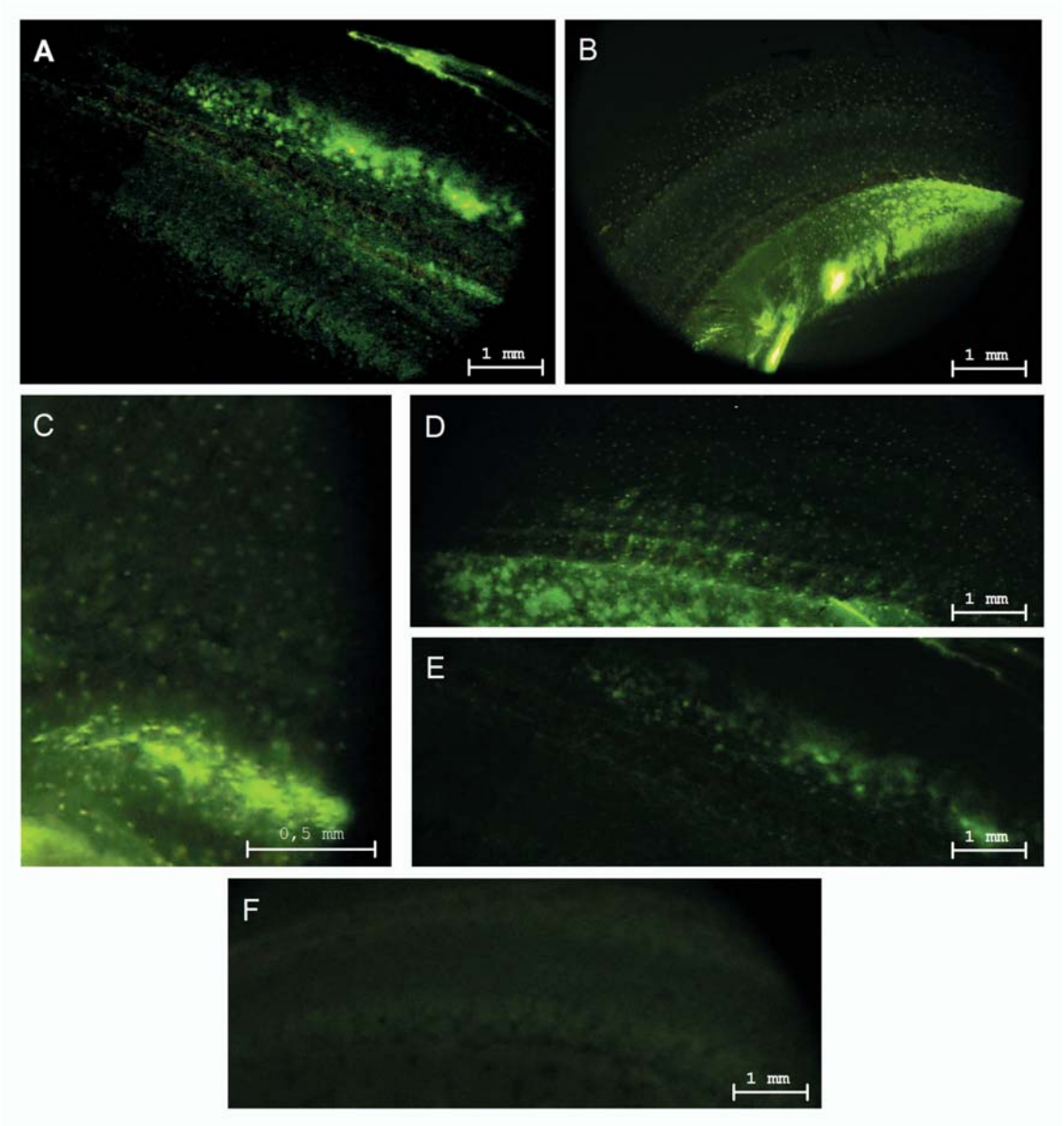

Figure 2. Enhanced green fluorescent protein (EGFP) expression in skin tissue of catfish larvae at 100 h post-hatching visualized by fluorescence microscopy (Olympus BX 51, Japan) (40x). **(A)** Negative control (not treated with exogenous DNA (pEGFP-N1 vector). **(B)** DR-treated group; **(C)** electroporated group; **(D)** group incubated with seminal plasma; **(E)** group incubated in the absence of seminal plasma; **(F)** negative control (not treated with exogenous DNA).

(7/90) of the animals expressing GFP. This can be explained by the presence of inhibiting proteins and DNases in the seminal plasma (data not demonstrated).

PCR analysis confirmed the presence of the transgene in DNA extracted from the different treatment groups, except the control group (figure 3). All control DNA samples were negative. The PCR product, corresponding to 500 bp of the pEGFP-N1 vector sequence, was detected in 8 of 20 (40%), 12 of 20 (60%), 5 of 20 (25%), 1 of 20 (5%) and 5 of 20 (25%) fish from the DR, DRE, E, INC and INCSP treatment groups, respectively. Foreign DNA was not detected in any of the 20 control fish examined. The difference in the PCR positivity rate between the DR and DRE, as well as between E and INCSP, and DR and E groups were not significant ($P>0.05$), but between the DRE and E groups it was highly significant ($P<0.05$) (table 1). PCR products obtained from the different treatments were confirmed by sequencing.

4. Discussion

In this study, we have shown that *Rhamdia quelen* spermatozoa can be successfully manipulated to generate transgenic fish, after applying an osmotic differential followed by electroporation or not, or through short incubation with foreign DNA in the presence or absence of seminal plasma. However, important statistical differences were observed with all the treatments. We showed that treatments used in the present study might affect reproductive parameters. SM was increased in the DRE group when compared with the control and other treatment groups. In contrast, Kang *et al.* (1999) reported a slight reduction in SM after electroporation of spermatozoa. Indeed, TAD, FR and HR appear to follow a similar pattern as demonstrated by high a FR and HR after treatment with DRE. These results suggest that spermatozoa of silver catfish can tolerate electroporation after DR, but further studies must be conducted to elucidate this mechanism. In contrast, sperm morphology does not seem to be affected by the treatments. A small cell agglutination observed in the E treatment group may have been due to a technical artifact.

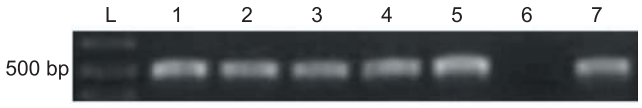

Figure 3. PCR detection of the 500 bp fragment of the green fluorescent protein (GFP) gene in purified genomic DNA from hatched *Rhamdia quelen* after different treatments. L, 1 kb DNA plus ladder; lane 1, DR; lane 2, DRE; lane 3, E; lane 4, INC; lane 5, INCSP; lane 6, negative control; lane 7, positive control. The reaction using genomic DNA of the control group as the template for PCR is the negative control and PCR with pEGFP-N1 as the template DNA is the positive control.

Kang *et al.* (1999) did not pursue the *in vitro* fertilization of eggs with non-electroporated sperm rehydrated in the presence of DNA and therefore they could not ascertain if an osmotic differential *per se* could be used to produce transgenic fish. One decade later, we report a novel approach without the electroporation step, which is more practical for mass production, since a larger number of gametes can be treated at one time just by an osmotic differential. We have demonstrated that electroporation is unnecessary, since there was no significant difference between the DR and DRE treatment groups ($P>0.05$), although a numerical difference favouring DRE was observed. Fluorescence microscopy, PCR and sequencing analysis results showed that the transgene entered the offspring cells following all the treatments. This agrees with the observations on other animal species and supports the idea that animal spermatozoa can be easily transfected *in vitro* (Lavitrano *et al.* 1989; Khoo *et al.* 1992; Gandolfi 1998; Spadafora 1998; Lavitrano *et al.* 2006; Hoelker *et al.* 2007; Lanes *et al.* 2009). However, it is important to consider that the success of transfection with foreign DNA seems to be a characteristic of the DNA, of the concentration, and the proportion of the linear and/or circular forms (Sciamanna *et al.* 2000; Shen *et al.* 2006; Hoelker *et al.* 2007). On the other hand, *in vitro* evidence suggests that sperm cells are able to acquire DNA and RNA molecules present in the medium (Gandolfi 1998; Magnano *et al.* 1998; Giordano *et al.* 2000; Sciamanna *et al.* 2009) but this has not been demonstrated under natural conditions.

Our study could not distinguish animal-to-animal variation in transgene EGFP expression and sperm potential, as has been described previously (Alderson *et al.* 2006). We did not evaluate transgene integration into the host genome and no animal was found to express EGPF in the entire body. We believe that the expression was transient and/or the integration event occurred after the one-cell stage embryo, producing mosaic animals. In SMGT, integration into the host cells occurs in a random manner (Wu *et al.* 2008). Recent studies have demonstrated high integration rates in birds (Harel-Markowitz *et al.* 2009), showing that this difficulty could be overcome in transgenic fish produced by SMGT in future.

The presence or absence of seminal plasma proteins strongly interferes with SMGT (Lavitrano *et al.* 1997; Lavitrano *et al.* 2006). In this study, it was possible to observe this through the results of the reproductive parameters. It has been demonstrated that the presence of seminal plasma protein is important for the maintenance of sperm quality in fish. However, we did not pursue the *in vitro* fertilization of eggs with sperm without seminal plasma submitted to the osmotic differential. It is conceivable that removal of proteins that block the entrance of foreign DNA and re-establishment of SM by the osmotic differential could favour transfection

rates. Lanes *et al.* (2009) inferred that DNA uptake by fish is strongly regulated by DNase in seminal plasma, a fact that is corroborated by our results, where DNA incubation in the absence of seminal plasma produced better results than DNA incubation with seminal plasma.

Transgenic fish offer an alternative to rodents as vertebrate laboratory animals. The use of fish in certain research areas can significantly reduce the exploitation of mammals, decrease costs and speed up the research process. In addition, fish can be useful for monitoring potential health hazards associated with exposure to chemicals in the aquatic environment (Lu *et al.* 2002; Hwang *et al.* 2004; Morita *et al.* 2004; Caelers *et al.* 2005; Houdebine 2005; Hu *et al.* 2006; Yazawa *et al.* 2006). In this sense, it is important to establish new transfer techniques *en masse* for genes of interest and those that are important in studies of molecular ecology and biotechnology (Hu *et al.* 2006).

In summary, our study demonstrated the transgene transmission of exogenous DNA into silver catfish larvae through SMGT technology.

Acknowledgements

This work was supported by the Brazilian Government through CNPq and CAPES. We are grateful to Dr Luis Antônio Suíta de Castro, Laboratório de Microscopia Eletrônica/Embrapa Clima Temperado – RS/Brazil and to Dr Luis Fernando Marins and Dr Sergio Noguez Piedras for technical support.

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MS received 18 December 2009; accepted 9 February 2010

*e*Publication: 24 February 2010

Corresponding editor: Seyed E Hasnain