Current Status of Sperm Cryopreservation in the Genus *Xiphophorus*

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Abstract

▼ Sperm cryopreservation is a useful technique for conservation programs aimed at preservation of endangered species and genetic resources in germplasm repositories. Fishes of the genus *Xiphophorus* provide important biomedical research models, specifically for melanoma studies, and are also used in basic research studies of evolution, toxicology, and endocrinology. Valuable broodstocks and specific *Xiphophorus* strains or lines have been created and need to be preserved in perpetuity. Although viviparous fishes are valuable research animals, many species are at risk of extinction because of human-induced activities such as water resource overexploitation, pollution, introduction of invasive fish species, and habitat alteration. For fishes with internal fertilization, sperm cryopreservation was essentially unexplored prior to the recent initiation of research on species of *Xiphophorus*. This review summarizes the current advances of sperm cryopreservation over the past five years within the genus, including the following topics: *1)* sperm characteristics and challenges posed for sperm cryopreservation by these unique characteristics; *2)* development of a practical protocol for sperm cryopreservation; *3)* artificial insemination using cryopreserved sperm for fertility detection, and *4)* the outlook for future research. The conclusions in this review may serve as a template for studies on sperm cryopreservation in all viviparous fishes.

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HBSS at an osmolality of 500 mOsmol/kg was also cryopreserved, and high motility (~55%) was obtained after thawing (Yang *et al.,* 2006). Parallel testing of cryopreservation of sperm from the same fish showed that motile and non-motile (immobilized) sperm had similar motility after thawing when activated in isotonic HBSS (Yang *et al.,* 2006). By immobilizing sperm prior to freezing with hypertonic HBSS at 500 mOsmol/kg, it was hypothesized that this procedure conserved sperm energy capacity, and consequently provided an advantage for artificial insemination compared to sperm cryopreserved using isotonic HBSS.

Dilution of sperm after collection was found to be necessary to maximize the suspension volume for subsequent experiments of various factor optimizations and multiplied use of each sample after cryopreservation. However, extreme dilution of sperm samples has also been found to reduce sperm motility in mammals (Harrison *et al.,* 1978), rainbow trout (Billard, 1983; Scott and Baynes, 1980) and oysters (Paniagua-Chavez *et al.,* 1998). Thus, dilution ratios of testis weight (mg) : HBSS (µl) with 1:50, 1:100, 1:200, 1:400, 1:800, and 1:1600 (v:v) were evaluated with sperm of *X. helleri.* Motility of sperm samples diluted at 1:50, 1:100 and 1:200 was found to be not significantly different before freezing or after thawing, but motility declined significantly at ratios higher than 1:200 (Huang *et al.,* 2004b).

Cryoprotectant Selection

Cryoprotectants are necessary for protection against freezing damage due to intracellular ice crystal formation and are usually grouped into two categories: permeating cryoprotectants (*e.g.* dimethyl sulfoxide (DMSO), methanol, and glycerol) and non-permeating cryoprotectants (*e.g.* egg yolk, milk, and proteins). The cryoprotectants used for sperm cryopreservation can be different across species (Fuller, 2004). For *Xiphophorus*, DMSO, N-dimethyl formamide (DMF), N-dimethyl acetamide, glycerol, propylene glycol (P-glycol), methanol, and sucrose were evaluated as cryoprotectants for sperm cryopreservation, each with final concentrations of 6% and 10% (v/v). The results from the sperm of *X. helleri* indicated that DMSO and glycerol were suitable cryoprotectants, and further evaluation of these two chemicals at different concentrations showed that glycerol was better than DMSO in retaining motility and prolonging storage time for *X. helleri* sperm after thawing, and the effective concentration for glycerol was 14% (Huang *et al.,* 2004c). Similarly, glycerol showed the best effect in cryopreservation of *X. couchianus* sperm with a concentration of 14% (Huang *et al.,* 2004a), and in immobilized sperm from *X. helleri* with concentrations of 10% and 15% (Yang *et al.,* 2006).

Prior to freezing, equilibration of cryoprotectants with sperm is necessary to allow the cryoprotectant to penetrate the cell. Equilibration times of 10, 20, 30, 60, and 120 min at 4 °C before cooling were evaluated for sperm of *X. helleri* and *X. couchianus.* An equilibration time of less than 30 min yielded the highest post-thaw motility in both species, but there was no consistent difference across equilibration times ranging from 10 to 120 min (Huang *et al.,* 2004a; Huang *et al.,* 2004c).

Packaging of Samples for Freezing

In sperm cryopreservation, packaging of samples for freezing and storage is important to standardize the cooling rate and assure sample identification. Because of the small volumes of sperm available, glass capillary tubes or cryovials have been employed in sperm cryopreservation in zebrafish and medaka (Aoki *et al.,* 1997; Draper *et al.,* 2004; Harvey *et al.,* 1982; Krone and Wittbrodt, 1997; Morris *et al.,* 2003). For *Xiphophorus* fishes, standard French straws used for sperm cryopreservation in livestock were chosen for sperm packaging with the smallest commercially available volume (0.25 ml). Compared to the capillary tubes or cryovials used for sperm cryopreservation in zebrafish and medaka, the use of French straws in *Xiphophorus* has the following advantages: potential for use with automated straw filling and sealing equipment, sample identification by permanent alphanumberic printing on straws, sample safety by complete sealing of the straw, and standardization of the cooling process.

Cooling Rate Selection

During the processes of freezing and thawing, sperm cells experience physicochemical changes such as formation of intracellular ice crystals, excessive dehydration and consequent concentration of solutes, and rapid osmotic swelling, which can be lethal conditions to sperm. Therefore, optimization of cooling rates, specific extenders, containers, and cryoprotectants is important, and this process is typically dictated by the interactions among these parameters.

Cooling rate is an important factor in sperm cryopreservation because it affects the osmotic balance of intracellular and extracellular solu-

tions during freezing. Theoretically, with an excessively slow cooling rate, osmotic equilibrium is maintained, and the freezable water leaving the cell can result in excessive dehydration; with an excessively fast cooling rate, little or no freezable water leaves the cell, and thus large intracellular crystals can form, causing damage to the cell. Ideally, a balanced situation allows survival when the cooling rate is fast enough to minimize the time of exposure to concentrated solutions and yet is slow enough to minimize the amount of intracellular ice formation. Optimum cooling rates vary with different cryoprotectants and the physiology of different species, and can be determined empirically by experimentation, or by theoretical calculation using techniques such as differential scanning calorimetry, a technique to measure heat release from the same cell suspension during freezing of live cells and dead cells (Devireddy *et al.,* 1998).

For *Xiphophorus*, cooling rates of 5, 15, 20, 25, 30, 35, and 45 °C per min from 5 °C to -80 °C were tested to freeze sperm before long-term storage in liquid nitrogen. The results showed that 20 °C to 30 °C per min was optimum when sperm were cryopreserved with 14% glycerol (Huang *et al.,* 2004a; Huang *et al.,* 2004c). The prediction by differential scanning calorimetry agreed with results in *X. helleri* (Thirumala *et al.,* 2005), but not in *X. maculatus* which the cooling rate was predicted as 47 °C/min (Pinisetty *et al.,* 2005).

Storage of Frozen Samples

Storage of cryopreserved samples at temperatures below -130 °C is necessary to assure long-term stability of samples (Rall, 1993). Thus, immersion of samples in liquid nitrogen (-196 °C) in a storage dewar is a standard method for cryogenic storage. During storage, the prime considerations are the identification and potential contamination of frozen samples. The use of plastic French straws for packaging offers the advantages of permanent labeling by automated printers, and the complete sealing of the straws which minimizes or prevents transfer of materials (*e.g.,* sperm cells or bacteria) among samples stored in the same dewar (Morris, 2005).

Thawing of Frozen Samples

Theoretically, damage that can occur during freezing also can occur during thawing (Leung, 1991) primarily through formation of ice crystallization. Thus, it is practical to thaw samples rapidly to minimize the period of vulnerability (Stoss, 1983). For *Xiphophorus*, a 5-sec exposure of frozen samples packaged in 0.25-ml French straws to a 40 °C water bath was suitable and did not affect motility or fertility after thawing (Huang *et al.,* 2004a; Huang *et al.,* 2004b; Huang *et al.,* 2004c; Yang *et al.,* 2006; Yang *et al.,* 2007b).

Current Protocol for Sperm Cryopreservation

Overall, based on post-thaw motility, the optimized protocol for sperm cryopreservation in *X. helleri* and *X. couchianus* is as following: *1)* collect sperm by crushing of dissected testis in HBSS at an osmolality of 310 or 500 mOsmol/kg with a volume of 20-100 times of testis weight. *2)* Add pre-made double-strength of HBSS-glycerol (28%) to the sperm suspension to produce a final concentration of 14%, mix well and equilibrate for 10-120 min at 4 °C. *3)* Load the sperm suspension into 0.25-ml French straws. *4)* Freeze the samples with a cooling rate of 20-25 °C/min from 5 °C to -80 °C. *5)* Plunge the samples into liquid nitrogen for long-term storage. *6)* Thaw the samples at 40 °C for 5 sec before using for insemination or further analysis.

Artificial Insemination with Cryopreserved Sperm

Xiphophorus fishes reproduce by internal fertilization. After insemination by the male, female *Xiphophorus* can store sperm for months, and can produce broods at approximately 30-day intervals for 4-5 months without the further presence of a male. The intervals between mating and the first few broods are irregular and vary from 26 to 90 days, but once a brood is produced by a female, the intervals between successive broods may become fairly regular (Tavolga, 1949). These features of *Xiphophorus* reproduction make artificial insemination with cryopreserved sperm challenging. Additionally, the body size of these fishes is small, and thus the injection of sperm for artificial insemination requires microscopy (10X or 20X magnification), and the injected sperm volume is limited to 5 µl.

Based on the reproductive features of *Xiphophorus*, the following strategies were necessary to evaluate the fertility of cryopreserved sperm by use of artificial insemination: *1)* Use of virgin females. *2)* Maintenance of inseminated females and monitoring of them for live young for 90 days. *3)* Use of females and males from different species for pa-

ternal confirmation by examination of offspring (interspecies hybrid broods produced through artificial insemination, such as *X. maculatus* × *X. helleri*, or *X. maculatus* × *X. couchianus* (Walter and Kazianis, 2001).

Challenges of Artificial Insemination with Cryopreserved Sperm

After development of practical protocols (Huang *et al.,* 2004a; Huang *et al.,* 2004c), sperm from *X. helleri* and *X. couchianus* were cryopreserved and thawed for artificial insemination to test fertility. Although high motility in thawed sperm was obtained, artificial insemination with cryopreserved sperm failed to produce live young in two initial trials (Dong *et al.,* 2005).

The first artificial insemination trial was attempted using females of *X. maculatus* with sperm from *X. helleri* and *X. couchianus*. Sperm were used immediately after thawing without further treatment. Sperm of *X. helleri* cryopreserved with 10% DMSO and *X. couchianus* cryopreserved with 14% glycerol were used, and no live young were observed after 90 days.

The second trial was designed to evaluate factors that could affect the success of artificial insemination, such as the sperm collection method and toxicity of the cryoprotectants. As stated above, the sperm used for cryopreservation were collected by crushing of the testis. Thus, the thawed sperm used for artificial insemination were single sperm cells rather than intact spermatozeugmata transferred during natural insemination (Hoar, 1969), and this could affect fertilization. In addition, cryoprotectants are often toxic (Fuller, 2004); for example, glycerol can decrease the motility of fresh zebrafish sperm from 90% to 4% after 30 min incubation at concentrations of 10% or 15% (Yang *et al.,* 2007a). The sperm injected for artificial insemination in *Xiphophorus* can be stored in the reproductive tract of the female for months prior to fertilization, and thus toxicity of glycerol could affect thawed sperm, the female, or oocytes. The results indicated that the collection method, the presence of the cryoprotectant (14% glycerol), and hybrid crossing were not responsible for the failure of artificial insemination, because fresh sperm from stripped and crushed testis with 14% glycerol produced live young. For cryopreserved sperm, no live young were produced from inseminated females of *X. helleri* or *X. couchianus*, even when the glycerol in the thawed sperm suspension was removed and the sperm concentrated by centrifugation.

Production of Hybrid Offspring with Cryopreserved Sperm from *X. helleri*

Considering the biology and biochemistry of sperm and the reproductive features in *Xiphophorus*, several hypotheses were proposed for the lack of production of live young with cryopreserved sperm in these first trials (Yang *et al.,* 2006), which included: *1)* after collection in HBSS at an osmolality of 310 mOsmol/kg, sperm were activated and motile during processing for cryopreservation, and this motility in the extender shortened the potential storage time of the sperm in the female reproductive tract prior to fertilization; *2)* the density of sperm used for artificial insemination after thawing was not sufficient for fertilization, or *3)* the toxicity of cryoprotectants used in cryopreservation shortened the sperm life or affected inseminated females or oocytes, and consequently affected fertilization. Based on these hypotheses, studies were designed to address these aspects. Previous studies had shown that: immobilization of sperm by hypertonic HBSS prolonged refrigerated storage; removal of cryoprotectant from thawed sperm increased motility retention to 72 h; and centrifugation at 1000× g for 5 min at 4 °C to concentrate sperm density did not decrease motility (Yang *et al.,* 2006). Also, the presence of glucose at 1g/L in HBSS was essential to retain motility duration for fresh and thawed samples, and the addition of 20% fetal bovine serum prior to freezing significantly increased the post-thaw motility compared to samples with 14% glycerol alone (Dong *et al.,* 2006).

Based on these findings, artificial insemination was performed with cryopreserved sperm of *X. helleri* and virgin females of *X. maculatus* (Yang *et al.,* 2007b). After thawing, all sperm samples were concentrated to a density of ~10^9cell/ml by centrifugation at 1000 *g* for 5 min at 4 °C. To determine the effect of immobilization of sperm on artificial insemination, motile sperm (in isotonic extender) and immobilized sperm (in hypertonic extender) were cryopreserved and used for insemination after thawing. To minimize the toxicity of glycerol in cryopreserved sperm, thawed sperm were washed with fresh HBSS by centrifugation at 1000 *g* for 5 min at 4 °C. In each case, successful fertilization occurred and live young were harvested. In one female, successive broods were produced at 55 d and 82 d after insemination. The paternity of the hybrid offspring via cryopreserved sperm was confirmed by analysis of distinctive phenotypes and genotypes (microsatellite markers). The hybrid offspring displayed the expected phenotypic traits that verified paternity (Figure 1).

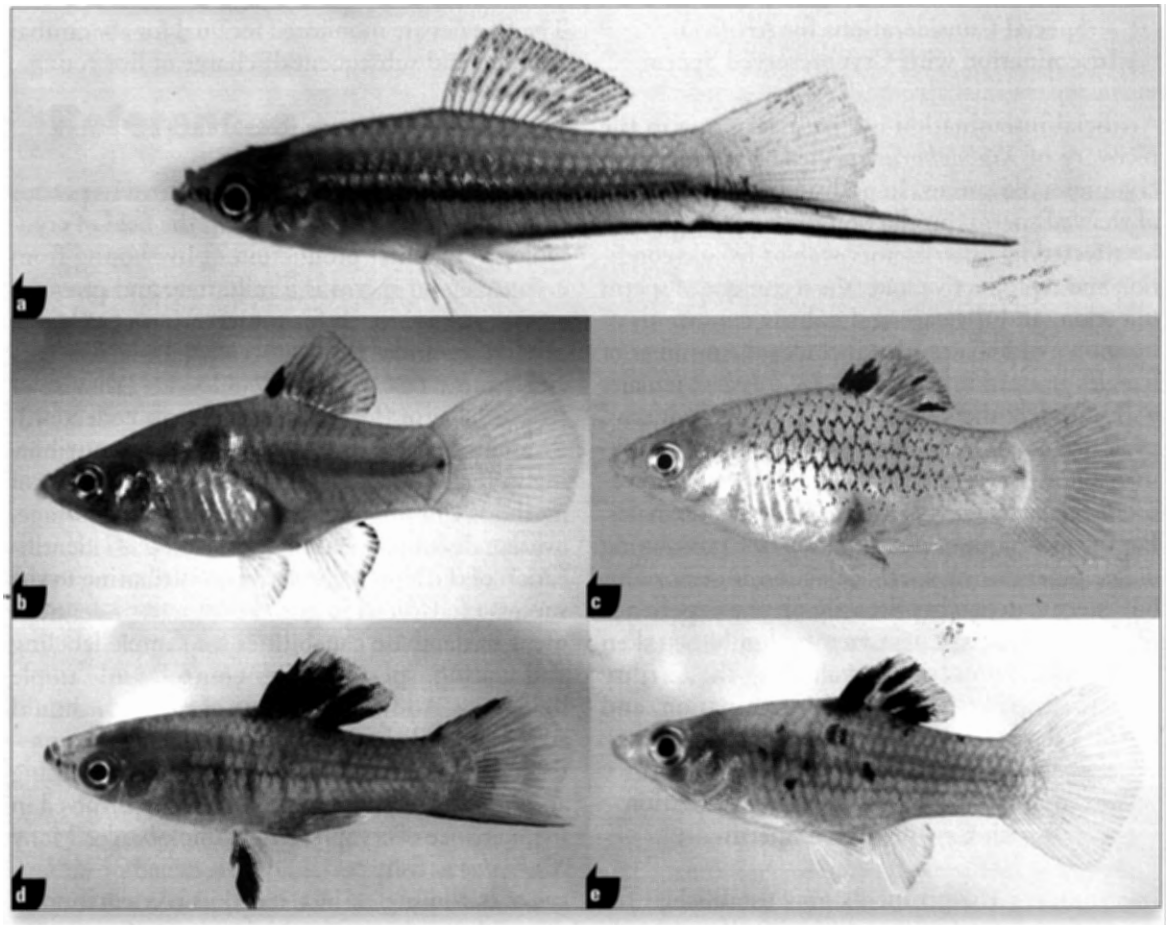

Figure 1.
Examples of parental and interspecies hybrid phenotypes. Examples of individual female (*d*) and male (*e*) interspecies hybrids from different broods (~10 months old) derived from artificial insemination of female *X. maculatus* Jp 163 A (B) with cryopreserved sperm samples collected from *X. helleri* (Sarabia) males (*a*). An example of a *X. maculatus* Jp 163 A male is presented in (*c*) (note absence of swordtail) to illustrate the differences between hybrid and parental pigment patterns. Note that the hybrids (*d* and *e*) exhibit enhancement of dorsal red (*Dr*) and the macromelanophore spotted dorsal pigment patterns compared to male or female *X. maculatus*. This F_1 phenotype has been well studied as a first step in the Gordon-Kosswig melanoma model. This cross absolutely requires artificial insemination to produce interspecies hybrid progeny, and thus verifies these broods resulted from insemination with cryopreserved sperm (figure modified from Yang *et al.,* 2007b)

The production of live young from cryopreserved sperm in isotonic and hypertonic extenders indicated that energy limitation was not a dominant consideration under the experimental conditions tested, although it could become important if sperm samples must be stored for several days prior to cryopreservation. Additionally, cryopreserved sperm samples from each male were divided into washed or non-washed treatments before injection into females, and in each case live young were produced. This indicates that removal of the glycerol from thawed sperm was not required under the conditions tested for successful artificial insemination.

This was the first report of successful fertilization and offspring production by cryopreserved sperm in viviparous fishes. The approach established in the studies above provides an effective means for genetic resource preservation in *Xiphophorus* fishes, and provides an example for comparable studies in other groups of viviparous fishes.

Production of Offspring with Cryopreserved Sperm in Other *Xiphophorus* Species

Based on the results from *X. helleri*, sperm from *X. couchianus* were cryopreserved in hypertonic HBSS buffer (~500 mOsmol/kg). After thawing, cryopreserved sperm were artificially inseminated into virgin females of *X. maculatus*. Hybrid offspring were harvested and the paternity was again confirmed via cryopreserved sperm from *X. couchianus* (Yang *et al.,* 2009). Subsequently, with the same protocol, cryopreserved sperm from two additional species (*X. maculatus* and *X. variatus*) have produced confirmed live young (our unpublished data).

Special Considerations for Artificial Insemination with Cryopreserved Sperm

Artificial insemination is a necessary step in the recovery of *Xiphophorus* species or strains from cryopreserved sperm. In addition to the viability of thawed sperm, production of live young could be affected by other factors such as female condition and reproductive state, the technique of sperm injection, and the care and culture conditions of inseminated females (this includes the number of females grouped in each tank). In *X. helleri*, females with different diets showed different reproductive performance (Chong *et al.,* 2004), and some females may be physiologically unprepared to reproduce, particularly some virgin or older females. For the technique of insemination, the positioning of the pipet during sperm injection is important, but success depends primarily on the experience of the technician. These factors should be taken into consideration when evaluating the fertility of thawed sperm by artificial insemination, and requires the use of necessary control treatments.

Current Protocol for Artificial Insemination with Cryopreserved Sperm

In summary, the protocols now established for sperm freezing, thawing, and post-thaw sample preparation for artificial insemination are as follows: *1)* Crush dissected testis in Hanks' balanced salt solution (HBSS) at an osmolality of 310 mOsmol/kg (HBSS310) or 500 mOsmol/kg (HBSS500). *2)* Mix sperm suspension with premade, double-strength glycerol-HBSS to a final concentration of 14% and equilibrate for 15 min. *3)* Cool the sperm suspension at a rate of 20 °C/min from 5 to -80 °C after loading of 240 µl in 250-µl French straws. *4)* Transfer the frozen samples to liquid nitrogen for storage. Before artificial insemination, *5)* thaw the samples at 42 °C for 5 sec in a water bath. *6)* Centrifuge (× 1000 g) the thawed sperm for 5 min at 4° C. *7)* Discard the supernatant and re-suspend in fresh HBSS (a wash step, recommended). *8)* Centrifuge to re-pellet the sperm and again discard the supernatant. *9)* Concentrate the sperm to a density of $\sim 10^9$ cell/ml. *10)* Inject 4-6 µl of concentrated sperm into individual, virgin females for artificial insemination. The females are monitored for 90 d for abdominal swelling and subsequent discharge of live young.

Future Research

Overall, sperm cryopreservation in viviparous fishes is a new area of research in the field of cryobiology. The first production of live young from cryopreserved sperm is a milestone and provides a practical approach for preservation of valuable genetic resources from other viviparous fish species. Future research could address: *1)* the use of stripped sperm (for non-lethal sample collection), *2)* identification of the major factors contributing to male-to-male variability, *3)* development of methods to monitor and evaluate sperm storage, ovarian development, and fertilization, *4)* identification of the female conditions contributing to the success of artificial insemination, and *5)* development of database capabilities for sample labeling and coding, sperm quality control, and sample inventory. Additionally, future research should address establishment of a high-throughput protocol for processing large numbers of samples using automated equipment, and biosecurity involved in maintenance of cryopreserved sample banks. Many *Xiphophorus* fish species are threatened or in danger of becoming extinct and thus conservation of the wild populations is a high priority. Eventually, the development of practical and high-throughput protocols for sperm cryopreservation and insemination can enhance the establishment of a conservation program for *Xiphophorus* fishes, and other viviparous fishes as well.

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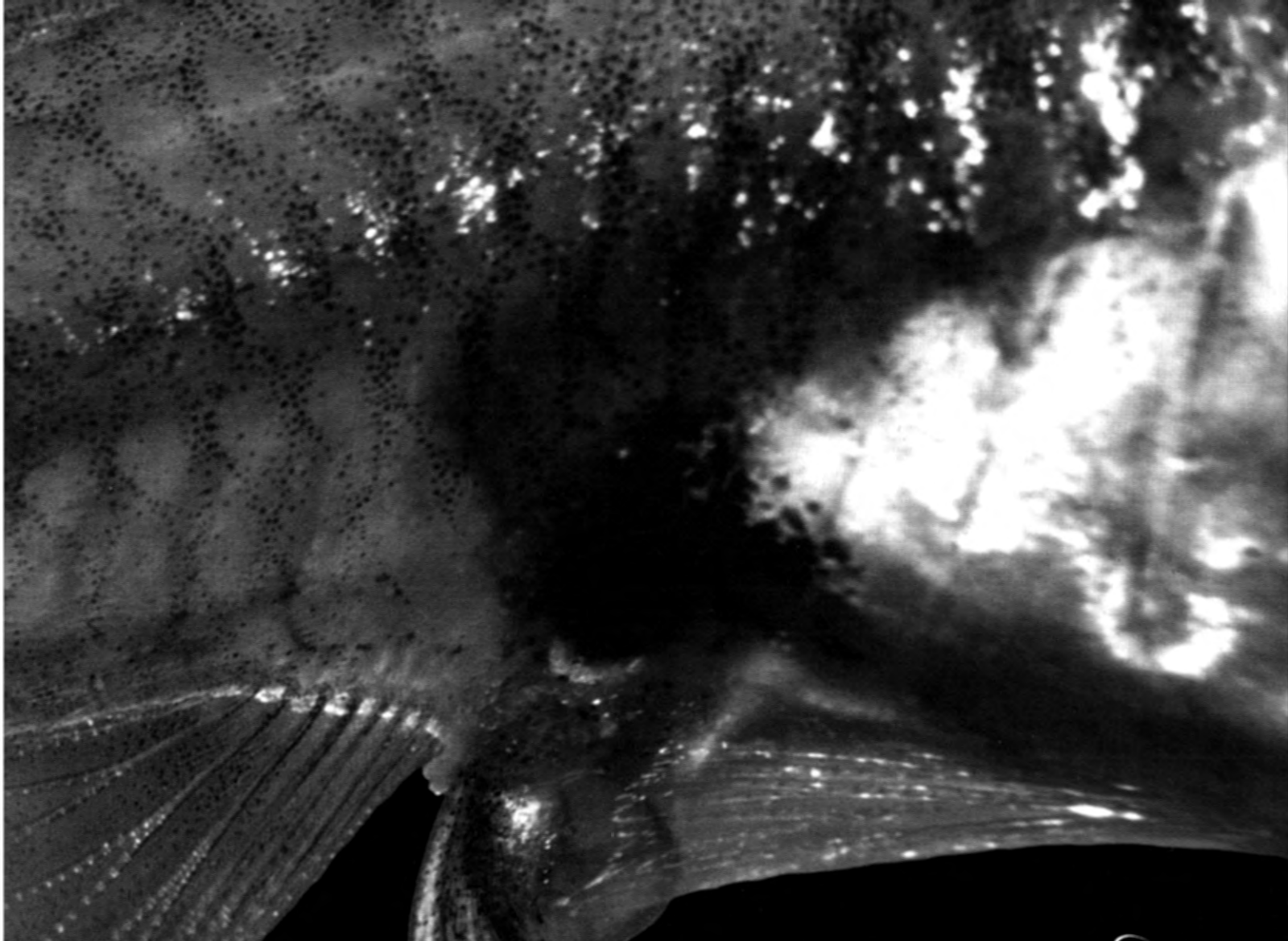

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