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Outcomes of *in vitro* fertilization with frozen-thawed sperm: An analysis of post-thaw recovery of sperm, embryogenesis, offspring morphology, and skeletogenesis for a cyprinid fish

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Running Title

Effect of cryopreservation on embryogenesis

Key words: cryopreservation; gene bank; embryology; early life history; larval morphology; deformity

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Abstract

Background: Gamete cryopreservation causes cellular damage and death. This study develops cryopreservation techniques for Levantine scraper, and deciphers how early offspring development is affected when eggs are sired with fresh and frozen-thawed sperm. Results: Cryopreserved sperm did not affect embryogenesis at 2- and 4-cell stages, but impaired embryonic development at 8-cell stage. Embryonic viability decreased at organogenesis, where only 34-49% of embryos showed viability with frozen-thawed sperm. Hatching success and percentage of normal hatched embryos declined when fertilized with frozen-thawed sperm. Considering only frozen-thawed cells the DMSO-5%, METH-5%, and METH-10% treatments yielded highest hatch, while METH-5% and PG-5% yielded the most normal hatched embryos. Larval spinal torsion was higher for fresh than frozen-thawed sperm, where larvae with spinal torsion showed vertebral fusion and shape alterations during exogenous feeding. Both fresh and cryopreserved treatments showed abnormalities in caudal skeleton, while rates of defective yolk-sacs were higher for cryopreserved sperm, where larvae with defective yolks showed oversized yolk extension. Percentage of larvae with defective heads/eyes were also higher for cryopreserved sperm. Conclusions: Results show how frozen-thawed sperm impairs embryonic/larvae development and identifies frequency and position of abnormalities. Future studies should investigate how sperm DNA damage may have caused these alterations.

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Introduction

Unfortunately, our aquatic biodiversity is declining, as more species are becoming threatened or extinct at an alarming rate (Gordon et al., 2018). This pattern will only increase as we see the results of global climate change in our oceans, rivers, and lakes. While little can be done to bring back lost species, we must work on securing the fate of others that are threatened or endangered, and at minimum have a repository of genetic information (i.e. germplasm repository) available as a safeguard; "buying us the time" necessary to improve aquatic habitats, if applicable. These repositories may also enable us to perform "genetic rescue," wherein genetically unrelated individuals from a larger population are infused into small, isolated populations that have inevitably lost genetic variability through inbreeding depression (Pimm et al., 2006; Fickel et al., 2007). Moreover, with the expansion of global aquaculture production (FAO, 2018) the capacity for long-term storage of cells (e.g. spermatogonia, sperm, blastomeres, or oocytes) can be pivotal for the creation of families and hatchery production (Butts et al., 2010; Martínez-Páramo et al., 2017; Hagedorn et al., 2002; Hagedorn et al., 2018).

Developing a cryopreservation protocol is no simple task, as it requires in depth knowledge of gamete physiology (Martínez-Páramo et al., 2017) and techniques must be designed specifically for each species or cell type (Cabrita et al., 2009). For instance, to cryopreserve sperm, water is typically extracted and replaced with antifreeze materials or

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a cryoprotectant agent (CPA), such as dimethyl sulfoxide (DMSO), dimethyl acetamide (DMA), methanol (METH), propylene glycol (PG), or glycerol at concentrations ranging from 5 to 20% (Labbé et al., 2013). There is no universal CPA or concentration for cryopreservation of aquatic germplasm and protective effects of CPAs vary between species. This is largely due to differences in CPA permeability and varying cell toxicity tolerance levels (Torres et al., 2016; Martínez-Páramo et al., 2017). Nevertheless, a prerequisite for germplasm cryopreservation is high CPA efficiency to prevent cell damage during freezing and thawing. Overall, these CPAs assist in the prevention of cryo-injuries during freezing and thawing but may be toxic when their concentration and/or exposure time is sub-optimal (Christensen and Tiersch, 1996; Yang et al., 2010; Best, 2015). Therefore, suitable CPA concentrations with minimum toxicity are needed.

Adding to complications, is that cryopreservation involves a series of chronological steps (Tiersch, 2011), each of which are highly impacted by interactions (Babiak et al., 2001; Butts et al., 2010) and may cause damages (Cabrita et al., 1998; Cabrita et al., 2001) to cell ultrastructure (Lahnsteiner et al., 1992), plasma membrane integrity (Yang et al., 2016), mitochondrial activity (Figueroa et al., 2017), and DNA integrity (Pérez-Cerezales et al., 2010). Unfortunately, limited studies have examined the long-term consequences of cryopreservation on developmental competence during the "critical" early life history stages (i.e. egg to first-feeding larvae), which typically have an unusually high-degree of mortality (Yúfera and Darias, 2007). As such, further knowledge at these later developmental stages is urgently needed.

After an egg is fertilized, early embryonic development continues with a series of mitotic cellular divisions, which form a blastodisc composed of symmetrically arranged blastomeres (Babin et al., 2007; Han et al., 2010). Several cleavage abnormalities may occur during embryogenesis, i.e. differences in blastomere shape and size or asymmetrically arranged blastomeres (Shields et al., 1997; Avery et al., 2009). Such cleavage abnormalities may adversely affect subsequent development, resulting in

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embryo mortality or larval abnormalities (Mazorra et al., 2003; Rideout et al., 2004; Avery and Brown, 2005), such as yolk-sac deformities, incomplete eye and body pigmentation, cranial and jaw anomalies, and notochordal shortening and curvatures (Boglione et al., 2001; Boglione et al., 2013a). Ultimately, this may impair growth and survival of larvae (Boglione et al., 2013a). As such, deformity/malformation rates may be used as an efficient tool to estimate the developmental potential of progeny derived from frozen-thawed sperm (Horváth and Urbányi, 2000; Miskolczi et al., 2005; Goes et al., 2017).

Here, we use Levantine scraper, *Capoeta damascina* (Valenciennes, 1842) as our experimental organism. Levantine scraper is a rheophilic freshwater cyprinid fish and has a wide distribution from Eastern Europe to West Asia (Coad, 2010). Due to overfishing, pollution, and habitat destruction, Levantine scraper populations are experiencing population declines. As such, studies are now being conducted to promote aquatic diversification/conservation, via artificial reproduction and captive rearing, for this societal and economically important species (Zadmajid and Butts, 2018; Zadmajid et al., 2019). Storage of germplasm using cryopreservation will be an important step for supporting these initiatives.

The objective of this study was to develop cryopreservation techniques for Levantine scraper, and decipher how embryogenesis or progeny development is affected when eggs are sired with either fresh and frozen-thawed sperm. Our results are expected to contribute baseline information on how *in vitro* fertilization using frozen-thawed sperm may impair embryonic and larvae development, in addition to identifying the frequency and positions of these abnormalities/deformities.

Results

Experiment I. Evaluation of diluents on sperm motility

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Before cryopreservation, cells are mixed with a buffered diluent of variable composition which prevent sperm motility activation, however, there is a pronounced species-specificity in diluent requirements (Labbé et al., 2013). Here, we tested the effect of two diluents (HBSS and MKS, see *Experimental Procedures*) on sperm motility at two time points (0 h and 24 h post-activation). The time × diluent interaction was significant for sperm motility ($P < 0.0001$), as such the model was revised into individual one-way ANOVA models at each post-activation time. At 0 h, diluents had no impact on sperm motility (ranged from 79.2 ± 1.1 to 82.9 ± 1.1%; $P = 0.056$), while at 24 h the diluents had an impact, where fresh-control (71.1 ± 1.7%) and HBSS (74.0 ± 1.7%) sperm had higher motility than MKS (59.0 ± 1.7%).

Experiment II. Acute toxicity of CPAs on sperm motility

For cryopreservation of aquatic germplasm, a suitable CPA concentration with minimum toxicity is needed (Torres et al., 2016). In the present study, to select a CPA concentration and appropriate equilibration time, acute toxicity of CPAs on sperm motility were analyzed. The time × extender (diluent + CPAs) interaction was significant for sperm motility ($P < 0.0001$; Fig. 1a-e) and longevity ($P < 0.0001$; Fig. 1f-j). Furthermore, all revised models (from 0 to 60 min post-incubation) were significant for sperm motility ($P < 0.0001$) and longevity ($P \leq 0.0008$). Specifically, at 0 to 60 min post-incubation, sperm motility always decreased when the cells were incubated with DMSO-10%, METH-10%, or PG-10%. DMSO-5% also caused a decrease in sperm motility at 0 and 15 min post-incubation. The METH-5% and PG-5% treatments had motility values (>80%) that were similar to the fresh-control (Fig. 1a-e). Sperm longevity declined after 0 (Fig. 1f), 15 (Fig. 1g), and 45 min (Fig. 1i) post-incubation for all treatments, while at 30 (Fig. 1h) and 60 min (Fig. 1j) the DMSO-5%, METH-5%, METH-10%, and PG-5% treatments were similar to the fresh-control.

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Experiment III. Evaluation of CPAs on post-thaw sperm motility

We examined the effect of CPAs on the quality of post-thaw sperm of Levantine scraper by assessing the percentage of motile cell and spermatozoa longevity. Relative to the fresh-control, post-thaw motility declined for all CPAs ($P < 0.0001$; Fig. 2). However, when considering only the frozen-thawed cells, the METH-5% treatment had the highest motility. For longevity, a two-fold decrease in sperm performance was detected after cryopreservation for all CPAs ($P < 0.0001$; Fig. 2).

Experiment IV. Effect of CPAs on in vitro fertilization, embryonic development and hatch success

Impaired fertility of cryopreserved sperm occurs from a combination of cell damage or lower post-thaw viability (Cabrita et al., 2005). After fertilization, we compared cell cleavage symmetry in early embryogenesis, produced using fresh and cryopreserved milt to determine whether sperm cryopreservation negatively influenced early development. Therefore, the asymmetrical pattern of blastomeres within developing embryos at the 2- to 8-cell stages of development was used as an indicator of embryo abnormality. Further, we tested whether asymmetric cleavages have a negative effect on the survival rate of embryos during segmentation and hatch success. Fertilization success was high for the fresh-control sperm (95.7%) but declined when eggs were fertilized with frozen-thawed sperm ($P < 0.0001$; Fig. 3). However, when considering only the frozen-thawed cells, the DMSO-5%, METH-5%, and METH-10% treatments had the highest fertilization success. Cryopreservation modified embryonic development by affecting cell division patterns during early development, resulting in high embryonic mortality during organogenesis (segmentation). At 2-cell ($P = 0.372$; Fig. 4) and 4-cell stages ($P = 0.502$; Fig. 4) the CPA treatments had no harmful effect on embryonic development, where >96% of embryos showed normal morphology (symmetric division). However, at the 8-cell stage, embryos fertilized with frozen-thawed sperm started to show a higher incidence of morphological

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abnormalities (asymmetric division or unequal blastomeres) in all CPA treatments when compared to the fresh-control ($P < 0.0001$; Fig. 4). By the time the embryos reached the organogenesis stage, only 34 to 49% of them showed viability for the frozen-thawed treatments, compared to 96% viability for the fresh-control ($P < 0.0001$; Fig. 4). Hatch success declined when eggs were fertilized with frozen-thawed sperm ($P < 0.0001$), but when considering only the frozen-thawed cells, the DMSO-5%, METH-5%, and METH-10% CPA treatments had highest hatch success (>39% hatch, Fig. 4).

Experiment V. Effect of CPAs on larval morphology and skeletogenesis

Larvae abnormalities would be expected to result from genetic mutations of key developmental genes (Wagner et al., 2005). However, the possible effects of sperm cryoinjury on offspring morphogenesis are scarce. We assumed that larvae morphogenesis measured in this study were sensitive enough to detect potential sperm cell damage induced by frozen-thawed process, and, if so, which phenotype were most greatly influenced. Here we showed, relative to the fresh-control, the percentage of normal hatched embryos was significantly lower for all CPA treatments (Fig. 5). However, when considering only the frozen-thawed cells, the METH-5% and PG-5% treatments yielded the highest percentage of normal hatched embryos (Fig. 5). The frequency of malformations is shown in Figs. 6 and 7a-t. The percentage of larval spinal cord torsion was significantly higher in the fresh-control than the CPA treatments ($P < 0.0001$; Fig. 6), where hatched larvae with spinal cord torsion, showed vertebral fusion and alterations in shape during the exogenous feeding period (Fig. 8b). Incidences of larval caudal fin torsion did not differ between treatments ($P = 0.2345$; Fig. 6), however, larvae with caudal fin torsion in both the fresh-control and the CPA treatments showed abnormalities in the caudal skeleton (Fig. 9b, c) or loss of caudal skeleton structures as well as the absence of caudal fin proximal radials during exogenous feeding period (Fig. 8b). Incidences of defective yolk-sacs was significantly higher in the CPA treatments

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than the fresh-control ($P < 0.0001$; Fig. 6), where larvae with a defective yolk-sac, developed yolk-sac edema or oversized yolk extension (Fig. 7b,e,l). Relative to the fresh-control, the percentage of larvae with defective heads was significantly higher in the CPA treatments ($P < 0.0001$; Fig. 6), where deformities were present in craniofacial structures, mainly the splanchnocranium or visceral skeleton (Fig. 10b,c). Moreover, incidences of larvae with defective eyes were higher in the CPA treatments than the fresh-control ($P < 0.0001$; Fig. 6). Here, larvae had smaller eyes and retinal pigmentation was not observed, even during retinal differentiation at 2 dph (Fig. 7e,f).

Experiment VI. Effect of CPAs on larval survival and morphometry

Larvae deformities may compromise either the survival of the individuals or their growth rate. Therefore, we examined larval survival and growth (measures by TL) originating from fertilization with fresh and cryopreserved sperm during early life history stages (from hatch to early exogenous feeding period). The time × CPA interaction ($P = 0.649$) and CPA main effect (P = 0.404) were not significant for larval survival, whereas a slight, but significant ($P < 0.0001$), decline in overall survival was detected from 0 to 15 dph (100 to 97.6 % survival). Additionally, the time × CPA interaction ($P = 0.282$) and CPA main effect ($P = 0.901$) were not significant for larval length, however, larvae significantly increased in size from 8.38 ± 0.08 mm at hatch to 12.69 ± 0.08 mm at 15 dph.

Discussion

Even though cryopreservation has been extensively used for assisted reproduction, there is now a growing body of evidence that it can have damaging effects not only on gamete performance but also on developing progeny (Pérez-Cerezales et al., 2010; Fernández-Díez et al., 2015; Fernández-Díez et al., 2018). As such, we investigated this phenomenon in great detail to pinpoint specific types of damage caused by

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cryopreservation during the critical early life history stages for Levantine scraper. In our study, we conducted the Experiments I-V using pooled sperm sample (with motility >75%) to eliminate individual variability. We hypothesized that the spermatozoa cell from each individual had different resistance to the cryodiluent and resistance to freezing based on sperm membrane integrity and functionality, ATP content, and mitochondrial functionality (Cabrita et al., 2005). Therefore, male-to-male variations might influence the success of cryopreservation. In support of this hypothesis, in northern pike, *Esox Lucius*, egg fertilization with cryopreserved milt varied from ~6 to 96 %, depending on the male individual, while, pooled milt resulted in ~71% fertilization success (Babiak et al., 1997). In addition, males with "bad" cryopreservation capability require higher sperm to egg ratios to compensate for lower frozen-thawed quality, thus jeopardize the enhancement of sperm cryopreservation protocols (Butts et al., 2011). However, in some species like channel catfish, *Ictalurus punctatus*, no relationship was observed between pre-freeze motility and post-thaw motility for individual males when high quality milt was used for cryopreservation (Christensen and Tiersch, 2005).

Motility is a direct and convenient index to evaluate fresh or frozen-thawed sperm quality. Our results showed that motility and longevity of frozen-thawed sperm was reduced when compared to fresh sperm for all CPAs. Damage to frozen-thawed sperm flagella (Billard et al., 2002; Butts et al., 2010), DNA (Cabrita et al., 2005; Pérez-Cerezales et al., 2009; Figueroa et al., 2016), cytoplasm-membrane, and mitochondrial integrity (Cabrita et al., 2005; Cuevas-Uribe et al., 2011) have been proposed as contributing mechanisms. Although post-thaw sperm longevity was not different between the tested CPAs, motility in Meth-5% was ~63%, which is higher than the other treatments, suggesting it efficiently reduced cryoinjuries as demonstrated for other fishes (Tiersch et al., 1994; Lahnsteiner et al., 2002; Horváth et al., 2003; Tiersch et al., 2004; Lujić et al., 2017; Asturiano et al., 2017).

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In vitro fertilization trials are a necessary step for validating the effectiveness of sperm cryopreservation protocols due to close correlations between sperm motility and fertility (Lahnsteiner et al., 1998; Dziewulska et al., 2011). Damage to sperm structure during freezing-thawing may impact fertility capability of the spermatozoon (Lahnsteiner et al., 1992; Gwo et al., 1993). In the present study, fertilization success was significantly declined when eggs were fertilized with frozen-thawed sperm. However, METH-5% treatment showed ~54% fertilization success which is greater than other CPAs, and this may have attributed to higher percentage of motile post-thaw sperm achieved by METH-5% treatment. Nonetheless, we propose that these traditional motility/fertility assays do not necessarily provide enough information to identify specific damage caused by cryopreservation since sperm with genetic defects due to freezing-thawing processes are still able to fertilize eggs (Twigg et al., 1998). As such, assessment of early embryonic development (e.g. blastomere morphology) and/or viability at later stages (e.g. organogenesis stage) could be more beneficial to predict the quality of embryos derived from fresh and frozen-thawed sperm.

Here, we examined the percentage of normal embryos during cleavage and in later developmental stages to better predict which stage(s) are most detrimentally impacted by fertilization using frozen-thawed sperm. Our results revealed that decreases in the percentage of normal embryos began at the 8-cell stage and by the time the embryos reached segmentation, only 34 to 49% showed normal viability, which resulted in lower hatching success for the CPA treatments. These results clearly indicate that embryonic mortality occurred during organogenesis and prior to hatch. Sperm DNA damage (e.g. genetic damage to sperm nuclear and/or mitochondrial genomes) exceeding the normal capacity of zygotic repair may have caused these embryonic alterations (Speyer et al., 2010; Pérez-Cerezales et al., 2010; Fernández-Díez et al., 2018), particularly during organogenesis (e.g. from epibolia to somite stage) (Pérez-Cerezales et al., 2010). It has been demonstrated that immediately after fertilization, extensive remodeling of the

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oocyte- and sperm-derived genomes occurs (Latham and Schultz, 2001). As well, oocytes have the capacity to repair damaged DNA at this point in ontogeny, preferably prior to first cleavage (Kopeika et al., 2003; Pérez-Cerezales et al., 2010). However, if egg repair mechanisms are not sufficient (Aitken and Baker, 2006) when the rate of DNA fragmentation is high (Pérez-Cerezales et al., 2010; Fernández-Díez et al., 2018), negative alterations to the embryo will continue, affecting embryonic organogenesis and/or progeny performance. Abnormal cleavage can be used to estimate hatch success due to positive correlations between the percentage of normal cleavage and hatch (Kjørsvik et al., 2003). Similar to our results, abnormal cleavage patterns resulted in lower embryonic viability and hatch success in Atlantic halibut, *Hippoglossus hippoglossus* (Mazorra et al., 2003), haddock, *Melanogrammus aeglefinus* (Rideout et al., 2004), and yellowtail flounder, *Limanda ferruginea* (Avery and Brown, 2005). In addition, abnormal cleavage patterns have also been shown to influence metamorphosis and juvenile deformities in some teleosts (Kjørsvik et al., 2003; Hansen and Puvanendran, 2010).

Here, it was demonstrated that sperm cryoinjuries go beyond fertilization and affect progeny, perhaps via transcriptomic profiling (Fernández-Díez et al., 2015; Fernández-Díez and Herráez 2018) or elicit DNA mutations in the germ line for generations (Ni et al., 2014). In this study, similar deformities were observed for larvae from fresh and frozen-thawed sperm, however, the rate of deformities was higher for the CPAs treatments. In particular, we showed that incidences of spinal cord and caudal fin torsion were high in newly hatched larvae, not only among the CPAs treatments, but also within the control group. When these types of malformations became more pronounced, swimming was impaired and larvae showed erratic behaviors, such as swimming in circles or spontaneous twitching movements. Interestingly, while incidences of spinal cord torsion were high in the control group, this type of deformity was low amongst the CPA treatments, which is contrary to what we had expected. Nevertheless, we showed

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that spinal cord torsion disrupted vertebral centrum differentiation during early larval development, which later evolved into vertebral abnormalities, such as compressed and fused vertebrae in subsequent life stages. Regardless of the origin, vertebral fusion was frequently observed in the posterior most caudal region of the larvae, which is a frequent position for vertebral fusion. Several types of vertebral abnormalities have been observed in teleosts, including vertebral fusion, changes in the number of vertebrae, scoliosis, lordosis, kyphosis, and shortened caudal fin and body (Boglione et al., 2001; Alix et al., 2017). The fact that skeletogenesis and skeletal tissue differentiation occurs during these early life stages, means that any alterations in embryogenesis may impact molecular pathways involved in larval skeletal ontogenesis and morphogenesis (Darias et al., 2011; Boglione et al., 2013a). Such impairments appear to be mediated by genetic or epigenetic changes to DNA in the sperm nucleus (Mair, 1992; Sola et al., 1998). For example, DNA damage in sperm positively correlated with offspring mortality or abnormality rates in other fishes (Devaux et al., 2015; Santos et al., 2018). In addition, environmental condition and larval nutrition may also impact phenotypic plasticity and ontogeny of the skeleton (Boglione et al., 2013b). Notochord abnormalities are frequently observed in newly hatched larval (Koumoundouros et al., 1997). This may be induced by improper inflation of the swim bladder, where larvae hyper-activity access oxygen causing gradual bending of the notochord/vertebral axis (Chatain 1994; Boglione et al., 2013a). In our study, however, incidences of spinal cord torsion were detected before swim bladder inflation and at hatch, thus such impairments may relate to DNA damages to the sperm haplotype genome during freezing/thawing process.

We found that the proportion of newly hatched larvae with a defective yolk-sac, head, and/or eye were higher in the CPAs treatments than the control group. Here, cranium abnormalities were already detectable in newly hatched larvae in which jaws were protruding or reduced. Similar to larvae with spinal cord and caudal fin torsion, those with defective heads were also able to enter the exogenous feeding stage, but with

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deformities in dentary, pre- and maxillary elements. In larval fishes, skull abnormalities are also associated with rearing environment (Roo et al., 2010) and genetic background (Sawayama and Takagi, 2016; García-Celdrán et al., 2016). Nevertheless, when the degree of anomaly is low, larvae with this type of abnormality may be able to recover over time, since their jaws continue to grow throughout larval development (Alderdice and Velsen, 1971; Beraldo and Canavese, 2011; Amoroson et al., 2016). For example, in gilthead sea bream, *Sparus aurata* anomalies of the opercular complex recovered in ~61% of a larval population after 16 months (Beraldo and Canavese, 2011).

As mentioned above, limited studies have addressed the impacts of sperm cryoinjury on early developmental studies. However, when studies are available, sometimes the results have been contradictory. For example, Miskolczi et al. (2005) obtained haploid malformed African catfish, *Clarias gariepinus* larval from eggs fertilized with frozen-thawed sperm and suggested that cryopreservation damaged the genome. In Russian sturgeon, *Acipenser gueldenstaedtii*, the number of cells with chromosome aberrations were high for embryos fertilized with frozen-thawed sperm (Mirzoyan et al., 2006), while in silver catfish, *Rhamdia quelen*, cryopreservation had no impact on genetic variability of offspring, but the production of normal larvae decreased (Goes et al., 2017). On the contrary, neither fresh or frozen-thawed sperm had an impact on rates of larval malformations for other species (Linhart et al., 2000; Chereguini et al., 2001; Horváth et al., 2003), and when abnormalities were observed, they were not enhanced for frozen-thawed sperm (Labbe´ et al., 2001; Ottesen et al., 2012; Bernáth et al., 2018). Collectively, in our study, a high percentage of abnormally hatched embryos were observed in CPA treatments likely because the frozen-thawed sperm used for fertilization had a relative high risk of genetic abnormalities. In addition, observed larval deformities in the control group maybe a reflection of stimulation of ovulation using either hormonal induction or maternal mRNAs. In some species, spawning induction using exogenous hormones (e.g. GnRHa/LHRHa) has been showed to have a negative

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impact on egg quality, fertilization successs, and larvae quality (El-Hawarry et al., 2016). For instance, in mahseer, *Tor tambroides* (Azuadi et al., 2011), and African catfish, *Clarias gariepinus* (El-Hawarry et al., 2016) hormonal induction of ovulation resulted in ~25.2 and ~10.5% of deformed larvae, respectively. In addition, maternal mRNAs that accumulate in the oocyte during oogenesis (e.g. maternal contribution to the cytoskeleton) are essential for early embryonic development and are involved in embryonic germ cell formation (Lubzens et al., 2017). The specific contribution of maternally inherited mRNA to egg developmental competence has been characterized in many vertebrate species (Bouleau et al., 2014; Lubzens et al., 2017). Exogenous hormone therapies may modify egg mRNA abundance of specific genes by modifications to the egg transcriptome and subsequent normal embryonic development (Bonnet et al., 2007).

The data showed that CPAs did not affect larval survival, but a significant decline in overall survival was detected (e.g. from 0 to 15 dph), where many larvae with defective eyes and yolk-sac died during endo-exogenous feeding or shortly after commencement of first-feeding. In addition, growth rate of larvae (measured as TL) from the CPA treatments were similar to the control from hatch until 15 dph. The consequences of cryopreservation on larval survival was not also significant for a variety of fishes (Tiersch et al., 1994; Ottesen et al., 2012; Viveiros et al., 2012; Rahman et al., 2016).

In conclusion, although cryopreservation reduced gamete and progeny quality, negative effects can be minimized by choosing METH-5% cryoprotectant for Levantine scraper germplasm cryobanking. Results presented here validate the use of blastomere cleavage patterns or embryonic viability during organogenesis as a quick and easy tool for predicting the quality of embryo batches using frozen-thawed sperm. Further studies are needed to evaluate the effect of CPAs on molecular pathways involved in embryonic organogenesis or larvae skeletogenesis. Additionally, osteological studies would further our understandings of developmental processes leading to vertebral deformities.

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Experimental Procedures

Fish origin and husbandry

Sexually mature Levantine scraper (~4 years; n = 54; 40 males and 12 females) were caught from Qeshlaq River (ca. 35º33'N, ca. 47º08'E), Sanandaj, Iran during the peak of the reproductive season (early June 2018 at 18-20 °C). Average body weight and total length (TL) (± SEM) were 110.6 ± 5.1 g and 24.65 ± 0.46 cm for males and 152.5 ± 22.8 g and 25.9 ± 1.6 cm for females, respectively. The sex of each fish was determined by visual examination. Specifically, fish were determined as females based on abdominal swelling and males by expulsion of a minute drop of milt upon gentle pressure applied to the abdomen, anterior to the urogenital opening. Fish were transported in oxygenated tanks (~1 h) to experimental facilities of the Fish Biology Lab at the University of Kurdistan. Fish were stocked in 6 × 500 L flow-through indoor round fiberglass tanks (127 × 76 cm; n = 8-10 fish/tank), held at 19 °C under natural photoperiod (14 light: 10 dark), and mean dissolved oxygen of 8.1 ± 0.2 mg/L. All animal manipulations were conducted according to the guiding principles for the use and care of laboratory animals by the Ethical Committee for Animal Experiments of Iran Veterinary Organization (IVO, protocols 30301 and 30309; 2014).

Milt collection and analysis

Spermiation of wild-caught broodstock was induced by Ovaprim™ (Syndel Laboratories Ltd., Canada) injection at 0.25 mL/kg body weight (BW) (Zadmajid et al., 2018) under anesthesia (75-115 ppm clove oil, C8392; Sigma-Aldrich, Inc., St. Louis, MO, USA). Milt was stripped at 12 h post injection with 5-mL syringes by applying slight pressure to the abdomen. Sperm samples were temporarily placed into Styrofoam coolers containing crushed ice (~4-5 °C) and analyzed within ~1 h post-collection. Sperm density was determined using a Neubauer haemocytometer counting chamber (BOECO,

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Germany) according to methods described by Butts et al. (2012). Estimated densities were expressed as the number of sperm cells per mL of milt ($\times 10^6$ spz/mL). Cell counting was done visually using a compound Eclipse E200-LED light microscope connected to a video monitor.

Sperm motility was evaluated in an activation solution (AS) containing 50 mM NaCl, 20 mM Tris, pH 8.5 (110 mOsmol/kg) at a ratio 1:50 (milt: AS) (Hatef et al., 2010). To assess sperm motility (%), the movement was video-recorded within 10 s post-activation using a CCD video camera (Digital Sight DS-L2, Nikon, Japan) mounted on a compound Eclipse E200-LED light microscope (×400 magnification). A short sequence of the video file was analyzed by Quick PHOTO MICRO 3.1. Six motion tracks were analyzed per sample within 10 s post-activation and fifty sperm were measured from each frame. All video recordings were conducted at room temperature and the time interval between two frames was 50 ms. Sperm longevity was defined as the time from activation until 10% of sperm remain motile (Gage et al., 2004). All analyses were performed by the same observer to minimize subjective differences during motility evaluation.

Experiment I. Evaluation of diluents on sperm motility

Two diluents were tested: Hanks' balanced salt solution (HBSS; Yang et al., 2010) which consisted of 0.137 M NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 5.55 mM glucose and modified Kurokura solution (MKS; Magyary et al., 1996) which consisted of 62 mM NaCl, 134 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM $NaHCO_3$. All diluents were adjusted to pH 7.6 (measured by pH meter; model 713, Metrohm Ltd. CH-9101 Herisau, Switzerland) and osmolality of ~310 mOsmol/kg (measured by vapor pressure osmometer; model K-7000; KNAUER, Germany), which is the pH and osmolality of Levantine scraper seminal plasma. To ensure that these diluents did not activate sperm, pooled fresh milt (with a minimum of three males; n = 15 pooled samples) was diluted in

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HBSS or MKS solutions at a final concentration of ~1.4 × 10^6 sperm/mL. Sperm and diluents were mixed in glass test tubes (16 × 100 mm, Hilgenberg GmbH, Waldkappel, Germany) and motility was assessed immediately or after refrigerator storage (4 °C) for 24 h. Fresh sperm served as the experimental control.

Experiment II. Acute toxicity of CPAs on sperm motility

HBSS showed the best results for Experiment I, thus used for further experimentation. Three CPAs: METH, DMSO, and PG were used at final concentrations of 5% and 10% (v/v) in HBSS at 4 °C on ice. Pooled fresh sperm (n = 10 pooled samples with motility >75%) was mixed with HBSS + CPAs at a final concentration of ~1.4 × 10^6 sperm/mL for motility estimation at 0 (within 10 s), 15, 30, 45, and 60 min post-exposure (Yang et al., 2007; Cuevas-Uribe et al., 2011). Freshly collected sperm at the same concentrations with a CPA-free diluent (HBSS) served as the experimental control. All chemicals were reagent grade (Merck, Darmstadt, Germany).

Experiment III. Evaluation of CPAs on post-thaw sperm motility

Extenders (diluent + CPA) were prepared ~24 h prior to use and held in a refrigerator at 3-4 °C. Pooled sperm samples from eight males were placed in 10 mL glass test tubes (16 × 100 mm, Hilgenberg GmbH, Waldkappel, Germany) on crushed ice (4 °C) prior to freezing. Only samples showing high fresh sperm motility (>75%) were used for freezing. Sperm samples were diluted with HBSS at 5 or 10% METH, DMSO, and PG at a final concentration of ~1.3-1.5 × 10^6 sperm/mL in glass test tubes, gently inverted for 10–15 s and equilibrated at 4 °C on crushed ice for 10 min. The diluted sperm were then loaded into 0.5 mL cryogenic straws (IMV, France) using a micropipette and sealed with polyvinyl alcohol (PVA; Merck, Darmstadt, Germany). Thereafter, straws were deposited on a horizontal tray floating 3 cm above the surface of liquid nitrogen in a Styrofoam box (30 cm length × 20 cm width × 25 cm height), equilibrated for 3 min, removed with

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forceps, put into goblets, and immersed in liquid nitrogen for later transfer to cryotanks (Irawan et al., 2010). After a week of storage in liquid nitrogen the straws were thawed in a temperature-controlled water bath at 40°C for 13 s (Horváth et al., 2003). Straw tips were cut off and contents released into disposable micro-centrifuge tubes (BRAND GMBH + CO KG, Germany). Motility of each sample was recorded within 3 min post-thaw as described for fresh sperm (see Section Milt collection and analysis). Fresh sperm served as the experimental control.

Experiment IV. Effect of CPAs on *in vitro* fertilization, embryonic development and hatch success

For *in vitro* fertilization, both PG and DMSO at final concentrations of 10% were excluded from further experimentation due to no post-thaw sperm motility. Pooled sperm samples from eight males were diluted with HBSS containing 5 or 10% METH, and 5% DMSO or PG at a final concentration of ~1.3-1.5 × 10^6 sperm/mL. Cells were equilibrated in crushed ice at 4 °C for 10 min. Sperm samples were loaded into 0.5 mL cryogenic straws and sealed with polyvinyl alcohol. Straws were frozen and thawed, as above. To obtain ovulated oocytes, wild-caught females (n = 6) were induced by Ovaprim™ injections at 0.50 mL/kg BW (Zadmajid and Butts, 2018). After hormonal induction (~20-25 h), the genital region was thoroughly cleaned with tissue to avoid gamete contamination with faeces or urine. Eggs (1.88 ± 0.06 mm in diameter) were obtained by applying slight pressure to the abdomen. After collection, pooled eggs batches (n = 21; ~1000 eggs/batch) were fertilized with either fresh or thawed sperm in plastic bowls at 1:2 × 10^5 for all samples (Linhart et al., 2000). Fertilization was initiated by the addition of ~5 mL tank water (19 °C) and mixed for 60 s. After *in vitro* fertilization, ~100 to 150 fertilized eggs were spread onto 9-cm Petri dishes (Li et al., 2015) and incubated at 19 °C in a closed water system. A minimum of 150 randomly selected eggs per batch were assessed for fertilization success by observing embryonic

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development at the 16- to 64-cell stages (at 5-7 h post fertilization) under a compound microscope (Eclipse E200-LED, Nikon, Japan). The percentage of normal embryos at early cleavage stages (2- to 8-cell stages) and viable embryos at late development (organogenesis or segmentation stage) were determined morphologically under a compound microscope (Eclipse E200-LED, Nikon, Japan) equipped with a digital camera (Digital Sight DS-L2, Nikon, Japan) (Kimmel et al., 1995; Postlethwait et al., 2016; Zadmajid et al., 2019). Hatch success [(hatched embryos / number of fertilized eggs) × 100] was calculated at ~5-6 h after initial hatching.

Experiment V. Effect of CPAs on larval morphology and skeletogenesis

Pooled sperm (n = 18 pooled samples) were diluted with HBSS comprising 5 or 10% METH, and 5% DMSO or PG at a final concentration of ~1.3-1.5 × 10^6 sperm/mL and cryopreserved as described in Experiment III. To obtain embryos, pooled eggs samples (n = 21; ~500 eggs/batch) were collected from five hormonally treated females and fertilized with either fresh or thawed sperm (n = 3 eggs batch/fresh or thawed sperm) as described in Experiment IV. To assess the percentage of normal hatched embryos and malformation type, newly hatched larvae were collected (n = 100/batch) from the different treatments, anesthetized in clove oil (40 ppm), and digitally imaged under a compound microscope (Eclipse E200-LED, Nikon, Japan) equipped with a digital camera (Digital Sight DS-L2, Nikon, Japan) for observations of morphology. Larval malformations (i.e. spinal cord torsion, caudal fin torsion, defective yolk sac, defective head, and defective eye; Bernáth et al., 2018) were counted by the formula: larval deformity rate (%) = (no. of deformed larvae / total larvae observed) × 100.

To learn more about abnormalities or impairments during skeletogenesis, any deformed larvae with spinal cord or caudal fin torsion, as well as defective heads were collected during exogenous feeding (from 9 days post hatch (dph) onwards). These larvae were euthanized in clove oil at 40 ppm, fixed in neutral-buffered 4% paraformaldehyde

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for 12 h, and rehydrated through a graded decreasing ethanol series (80, 50, and 25%). Specimens were cleared in 0.5% KOH (Merck, Darmstadt, Germany), and then double stained with Alcian Blue (8GX, Sigma-Aldrich, Inc., St. Louis, MO, USA) to reveal cartilages and stained with Alizarin Red (Alizarin Red S, Sigma-Aldrich, Inc., St. Louis, MO, USA) to reveal calcified bones (Taylor and Van Dyke, 1985; Desvignes et al., 2018a,b). Cleared and stained larvae were stored in a solution of 70% glycerol (Merck, Darmstadt, Germany) and 0.3% KOH at 4 °C. Images were taken by a TrueChrome Metrics camera (TUCSEN, China) mounted on a compound microscope (GENUS, China). Images were processed with Corel Draw X6 software (Corel, Ottawa). The essential terminology of skeletal elements were based on definitions from Li et al. (2015), Conway et al. (2017) and Desvignes et al. (2018a,b).

Experiment VI. Effect of CPAs on larval survival and morphometry

Newly hatched larvae from Experiment V were reared in 75 L glass aquariums (n = 15; 76 cm length × 33 cm width × 33 cm height) in a flow-through freshwater system at a flow rate of 0.1 L/min. Aquaria were outfitted with an overflow sieve (250 mm mesh netting) positioned just beneath the water surface. Larvae were stocked at a density of 25 larvae/L and reared at 14 h light/10 h dark and ambient temperature (~19 °C). Water quality parameters were monitored daily: 8.1 ± 0.2 mg/L dissolved oxygen, 0.12 ± 0.02 mg/L ammonia, <0.0033 mg/L nitrite, and pH of 7.7 ± 0.2. During the endo-exogenous feeding period (6-8 dph; Zadmajid et al., 2019), larval were fed *Artemia* nauplii at 230 nauplii/L. From 9 dph (commencement of exogenous feeding period), *A.* nauplii were increased to 5,000 nauplii/L. For morphometric investigations, larvae were randomly collected from 1 to 15 dph and anesthetized in clove oil at 40 ppm. Larvae TL (n = 10/aquarium; 3 aquarium/treatment) were measured daily with a micrometer (Digital Vernier Caliper, China) at 5:00 pm. Daily mortalities were removed and recorded at 8-9 am and larval survival (%) was recorded from 1 to 15 dph (n = 10 replicate/treatment).

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Statistical analyses

All data were analyzed using SAS statistical analysis software (v.9.1; SAS Institute Inc., Cary, NC, U.S.A, 2003). Residuals were tested for normality (PROC UNIVARIATE; Shapiro-Wilk test) and homogeneity of variance (PROC GPLOT; plot of residuals vs. predicted values). Data were transformed to meet assumptions of normality and homoscedasticity when necessary. Treatment means were contrasted using the Tukey's least-squares means method. Error bars represent least square means standard error. Alpha was set at 0.05.

Evaluation of diluents on sperm motility were analyzed using a repeated measures factorial ANOVA model containing the time (0 and 24 h post-collection), diluent (i.e., fresh-control, HBSS, MKS), and time × diluent interaction. In the case of a significant interaction, the model was revised into individual one-way ANOVA models at each time. Acute toxicity of CPAs on sperm motility were analyzed using a repeated measures factorial ANOVA containing the time (0 to 60 min post-incubation), CPA (i.e., fresh-control, METH-5%, METH-10%, DMSO-5%, DMSO-10%, PG-5%, PG-10%), as well as the time × diluent interaction. Models were revised if significant interactions were detected. Post-thaw sperm motility, sperm longevity, fertilization success, embryonic development (i.e. 2-cell, 4-cell, 8-cell, and segmentation), hatch success, and larval malformations (i.e. spinal cord torsion, caudal fin torsion, defective yolk sac, defective head, and defective eye) were analyzed using a series of one-way ANOVA models. Larval survival and morphometry were analyzed using repeated measures factorial ANOVA models containing the time (0 to 15 dph), CPA (i.e. fresh-control, DMSO-5%, METH-5%, METH-10%, PG-5%), and time × CPA interaction. In the case of a significant interaction, the models were revised into individual one-way ANOVA models at each dph. If no interaction was detected the main effects were interpreted.

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Conflict of interest

Authors declare they do not have any conflicts of interest.

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Figure legends

Fig. 1. Effect of cryoprotectants toxicity on Levantine scraper, *Capoeta damascina* sperm motility (a-e) and longevity (f-j) from 0 to 60 min post sperm activation. Separate one-way ANOVA models were run at each time post-activation. Error bars represent least square means standard error (n= 10). $^{a–e}$Treatments without a common superscript differed ($P < 0.05$). Abbreviations: Control = fresh sperm; DMSO = dimethyl sulphoxide; METH = methanol; PG = propylene glycol.

Fig. 2. Effect of cryoprotectants on Levantine scraper, *Capoeta damascina* post-thaw sperm motility and longevity. Error bars represent least square means standard error (n= 18). $^{a–d}$Treatments without a common superscript differed ($P < 0.05$). Abbreviations: Control = fresh sperm; DMSO = dimethyl sulphoxide; METH = methanol; PG = propylene glycol.

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Fig. 3. Effect of cryoprotectants on Levantine scraper, *Capoeta damascina* post-thaw fertilization success. Error bars represent least square means standard error (n= 15). [a–c]Treatments without a common superscript differed ($P < 0.05$). Abbreviations: Control = fresh sperm; DMSO = dimethyl sulphoxide; METH = methanol; PG = propylene glycol.

Fig. 4. Embryonic development at 2-, 4-, 8-cell stages, segmentation, and hatch for Levantine scraper, *Capoeta damascina* using cryopreserved sperm with different cryoprotectants. Error bars represent least square means standard error (n= 15). [a–c]Treatments without a common superscript differed ($P < 0.05$). Photomicrographs are representative of normal symmetrically cleaving and abnormal asymmetrically cleaving embryos. Abbreviations: BM = blastomere; Control = fresh sperm; DMSO = dimethyl sulphoxide; METH = methanol; PG = propylene glycol; PV = perivitellin space.

Fig. 5. Normal hatched embryo for Levantine scraper, *Capoeta damascina* using cryopreserved sperm with different cryoprotectants. Error bars represent least square means standard error (n= 15). [a–c]Treatments without a common superscript differed ($P < 0.05$). Abbreviations: Control = fresh sperm; DMSO = dimethyl sulphoxide; METH = methanol; PG = propylene glycol.

Fig. 6. Larval deformities for Levantine scraper, *Capoeta damascina* using cryopreserved sperm with different cryoprotectants. Error bars represent least square means standard error (n= 15). [a–c]Treatments without a common superscript differed ($P < 0.05$). Representative photomicrographs are shown for visualizing each type of larval derformity. Abbreviations: Control = fresh sperm; DMSO = dimethyl sulphoxide; METH = methanol; PG = propylene glycol.

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Fig. 7. Morphologically normal (a, d, g, j, m, o, q, s) and phenotypic deformed (b, c, e, f, h, i, k, l, n, p, r, t) Levantine scraper, *Capoeta damascina* larval obtained from *in vitro* fertilization using frozen-thawed sperm. Abbreviations: CFT = caudal fin torsion; DE = defective eye; DH = defective head; dph = days post hatch; DYS = defective yolk sac; SCT = spinal cord torsion.

Fig. 8. Schematic representative dorsal view of Levantine scraper, *Capoeta damascina* larvae obtained from *in vitro* fertilization using frozen-thawed sperm. Shown in panel (a): normal larval at 11 days post hatch (dph). Shown in panel (b): larvae with spinal cord and caudal fin torsion (indicated by red lines) at 13 dph. Abbreviations: BA = branchial arches; CFT = caudal fin torsion; E = eye; PF = pectoral fin; SCT = spinal cord torsion; V = vertebrae.

Fig. 9. Schematic representative lateral view of caudal vertebrae of Levantine scraper, *Capoeta damascina* larvae obtained from *in vitro* fertilization using frozen-thawed sperm. Shown in panel (a): larvae with normal caudal vertebrae at 24 days post hatch (dph). Shown in panel (b): larvae with caudal skeleton torsion at 19 dph, with abnormality in the region of preural 2-5 of the caudal vertebrae (indicated by red lines). Shown in panel (c): larvae with caudal skeleton torsion at 17 dph, with apparent a supernumerary spine (pseudoneural spine) in the caudal domain. Abbreviations: CFT = caudal fin torsion; Epr = epural; Ha = haemal arch; Hpy = hypural; Pe = preural centra; Phy = parhypural; Ps = pseudoneural spine; pu1+u1+u2 = compound centrum made by the fusion of preural centrum 1 and ural centra 1 and 2; Urn = uroneural;

Fig. 10. Schematic representative lateral view of head skeletons of Levantine scraper, *Capoeta damascina* larvae obtained from *in vitro* fertilization using frozen-thawed sperm. Shown in panel (a): larvae with a normal head at 13 days post hatch (dph). Shown in

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panel (b): larvae with defective head at 13 dph with abnormality in dentary, pre- and maxillary (e.g. reduction in length; indicated by dotted lines). Shown in panel (c): larvae with defective head at 13 dph where dentary tip skewed off-centre and are not oriented parallel to the upper jaw (indicated by dotted lines). Abbreviations: Ang = angular; Cm = coronomeckelian,; Dent = dentary; Ec = ectopterygoid; En = endopterygoid; E = eye; Hm = hyomandibular; Io: interopercle; Ma = maxilla; Mc = Meckel's cartilage; Mt = metapterygoid; Op = opercle; Pa = palatine; Pm = premaxilla; Po = preopercle; Qu = quadrate; Ra = retroarticular; So = subopercle; Sy = symplectic.

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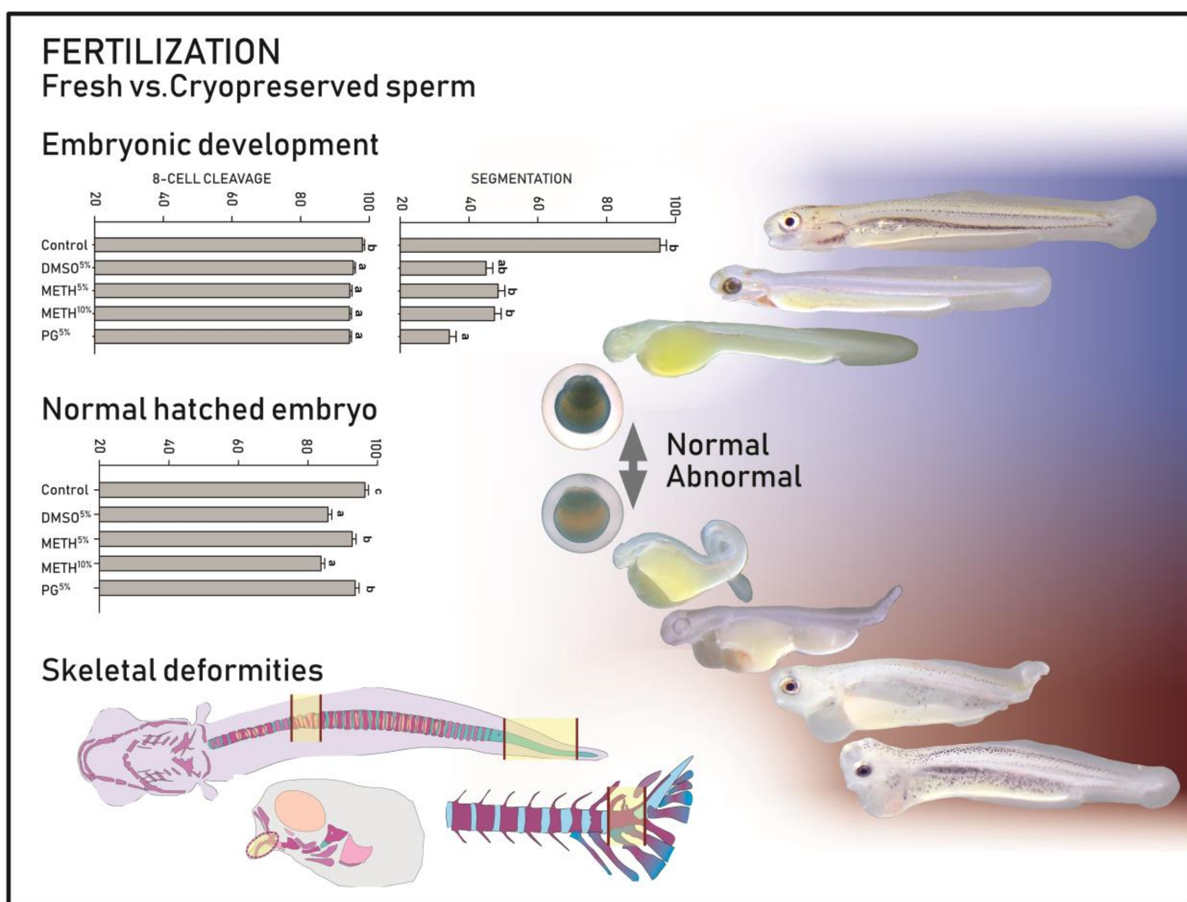
Following germplasm cryobanking for Levantine scraper, the impact of cryoprotectants on embryogenesis and early larvae quality were assessed. *In vitro* fertilization using frozen-thawed sperm impaired embryogenesis during early cleavage and organogenesis and caused abnormality in early larvae development



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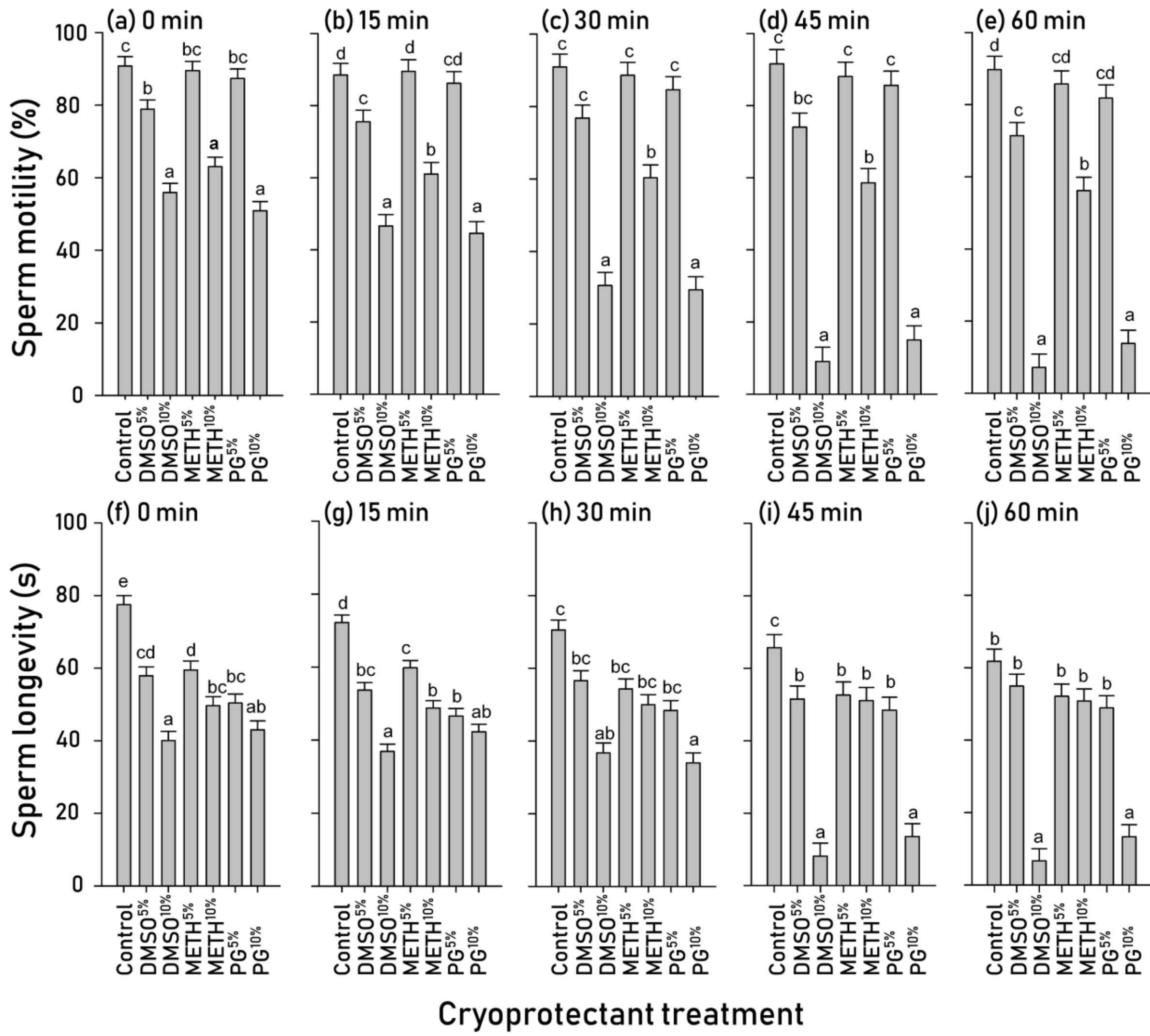

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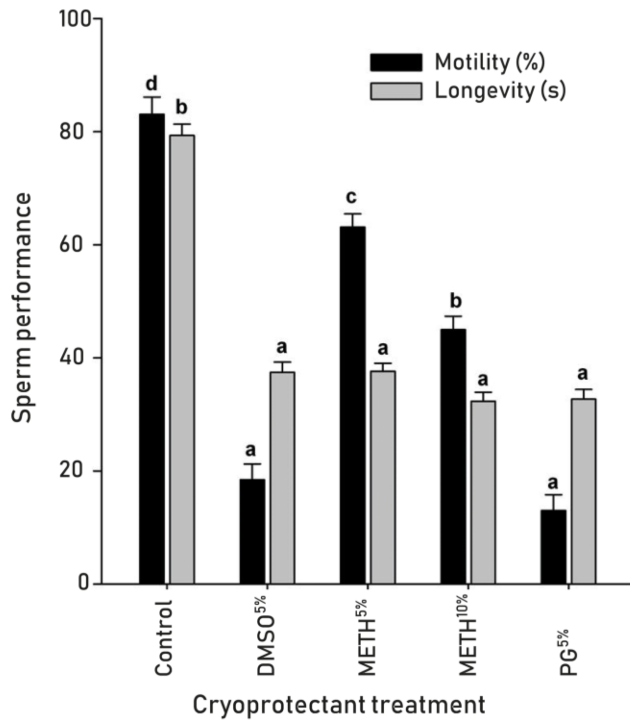

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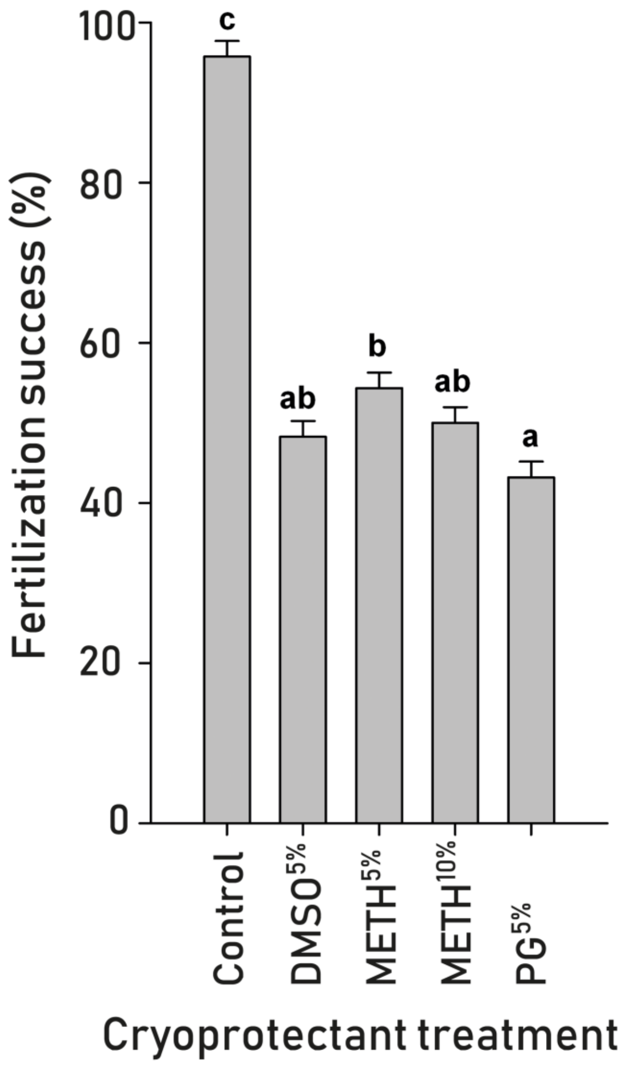

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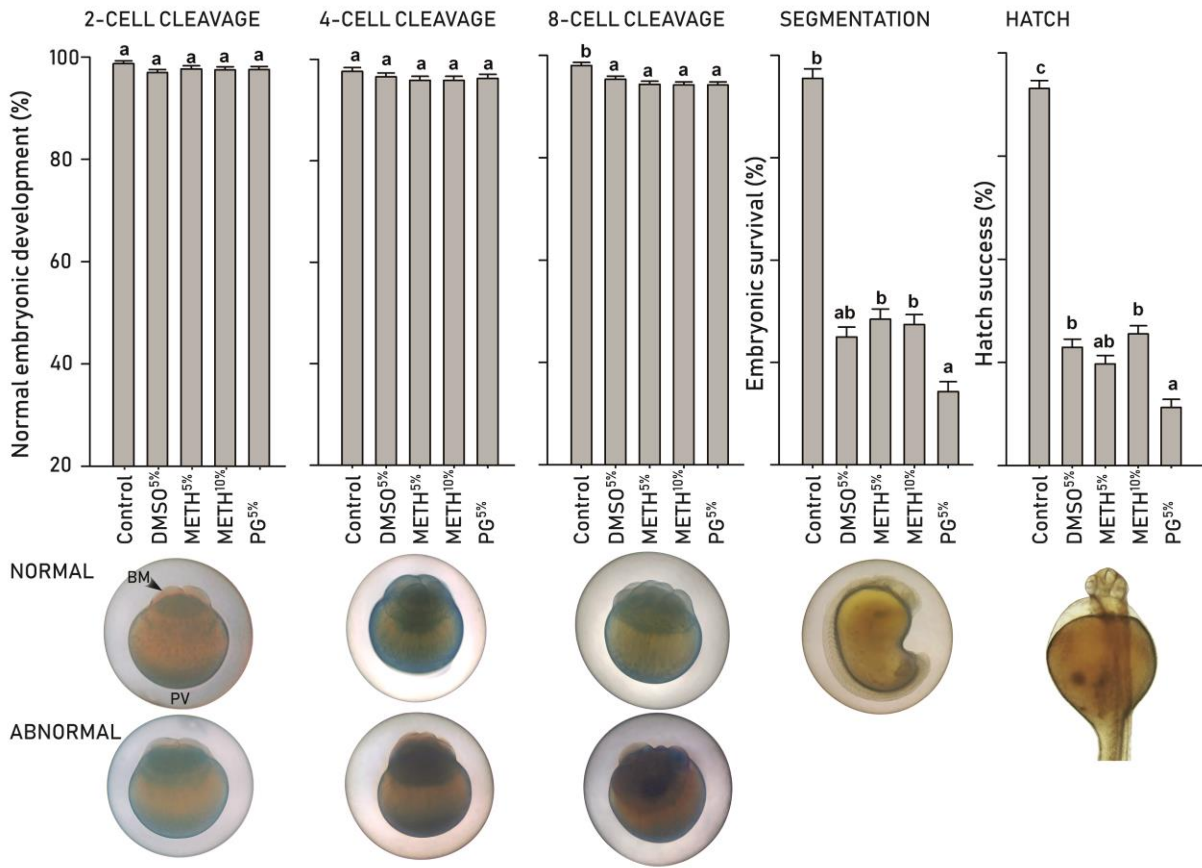

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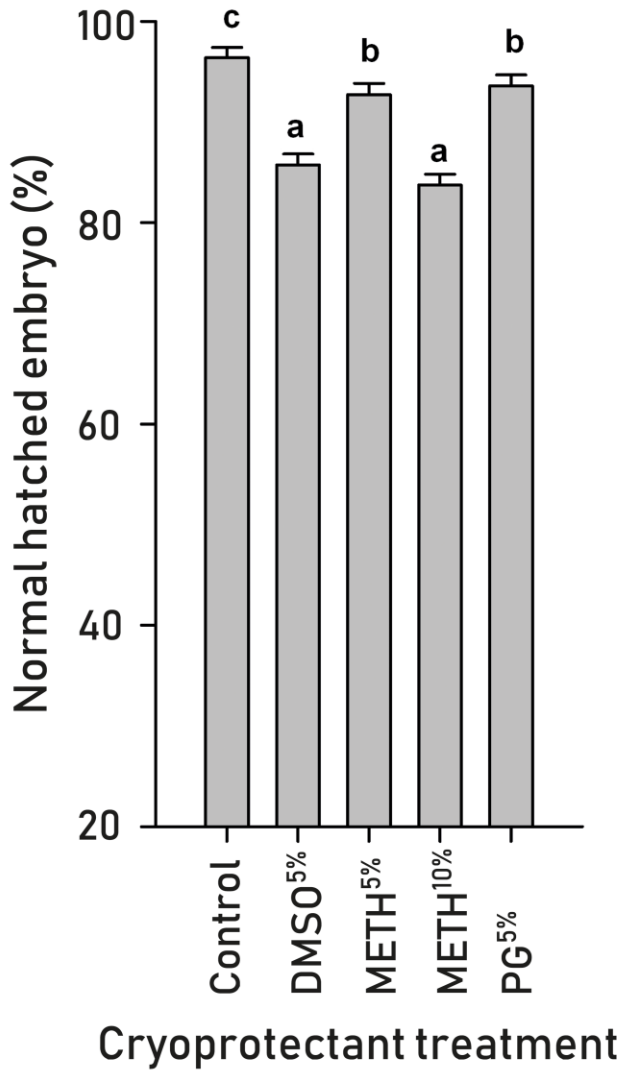

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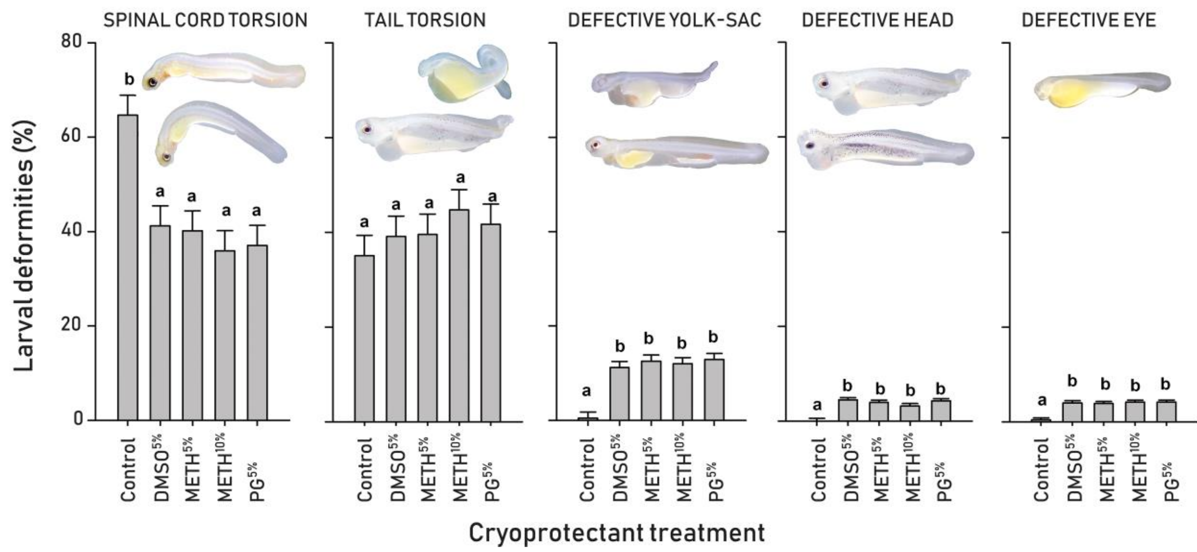

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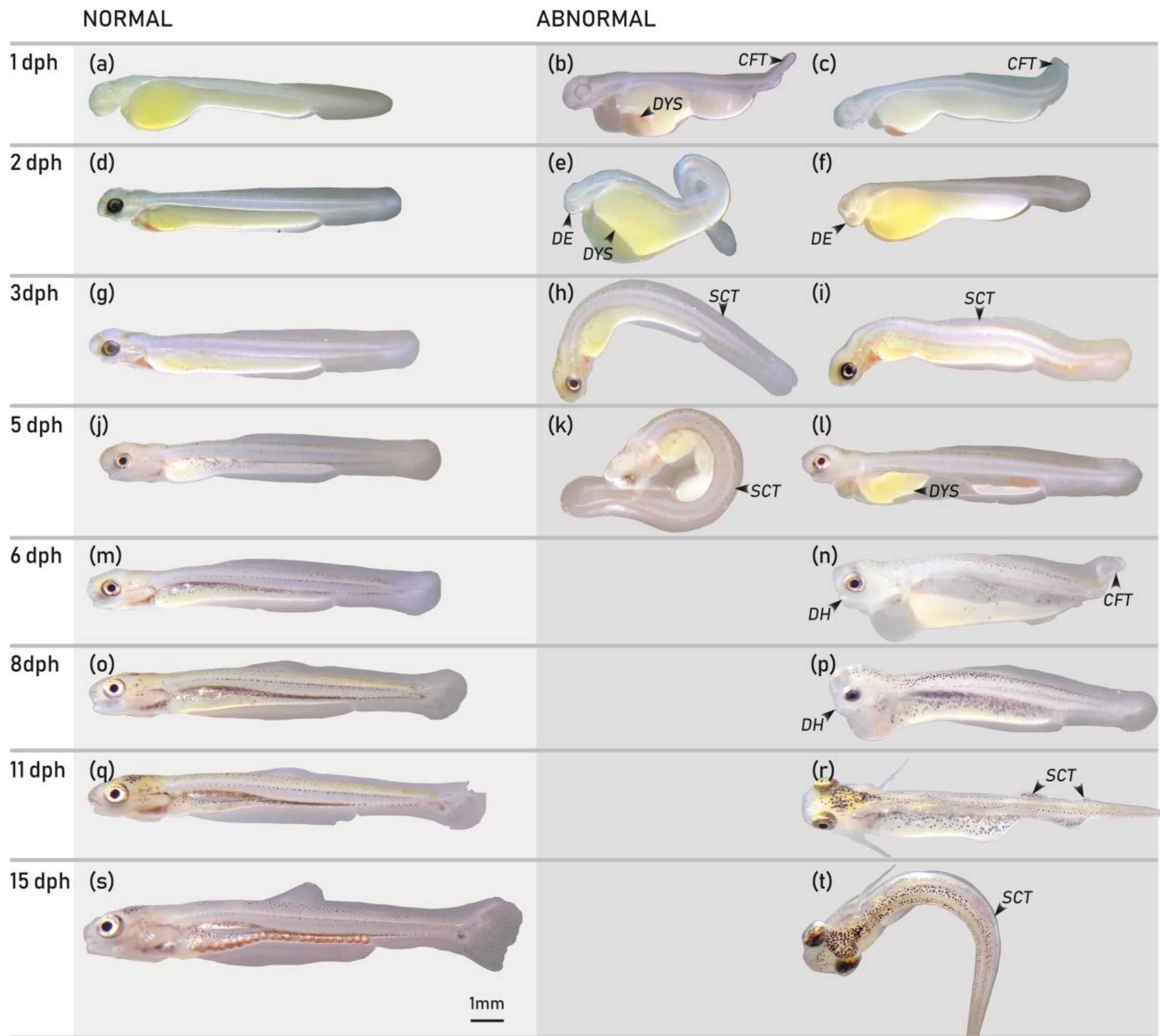

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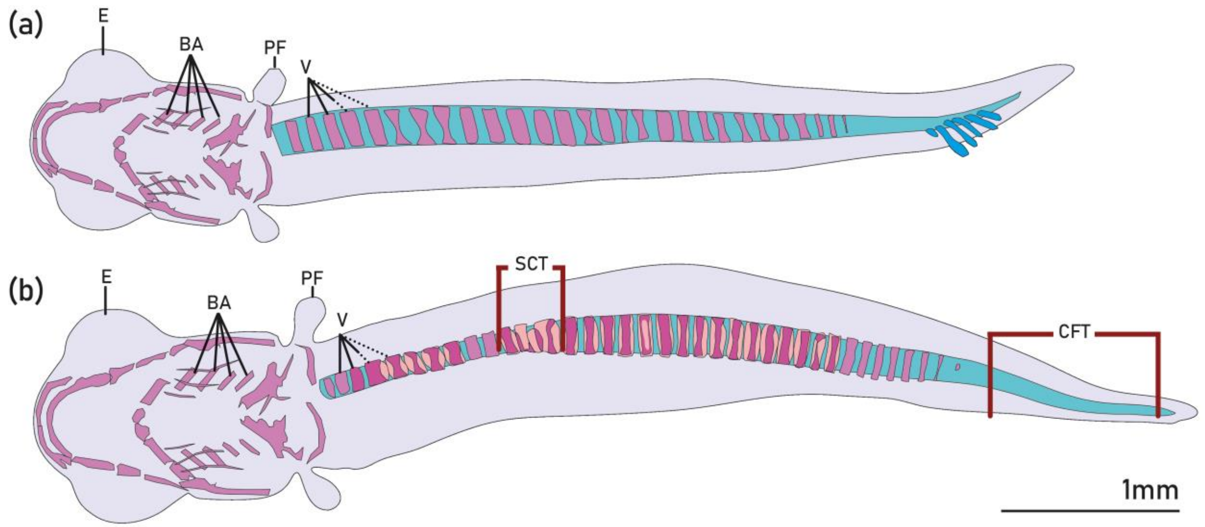

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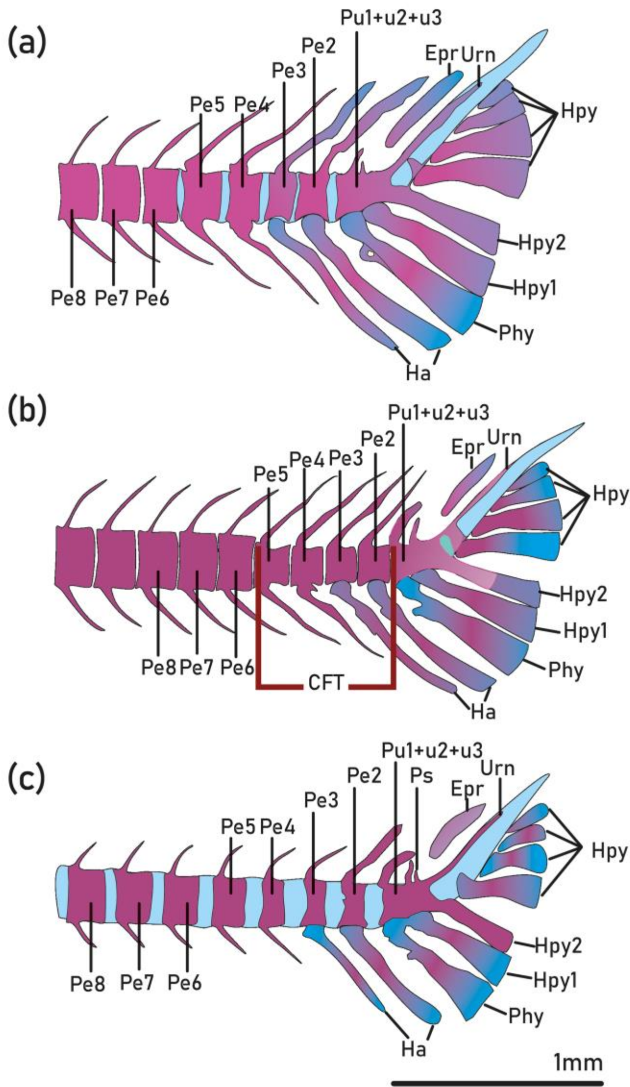

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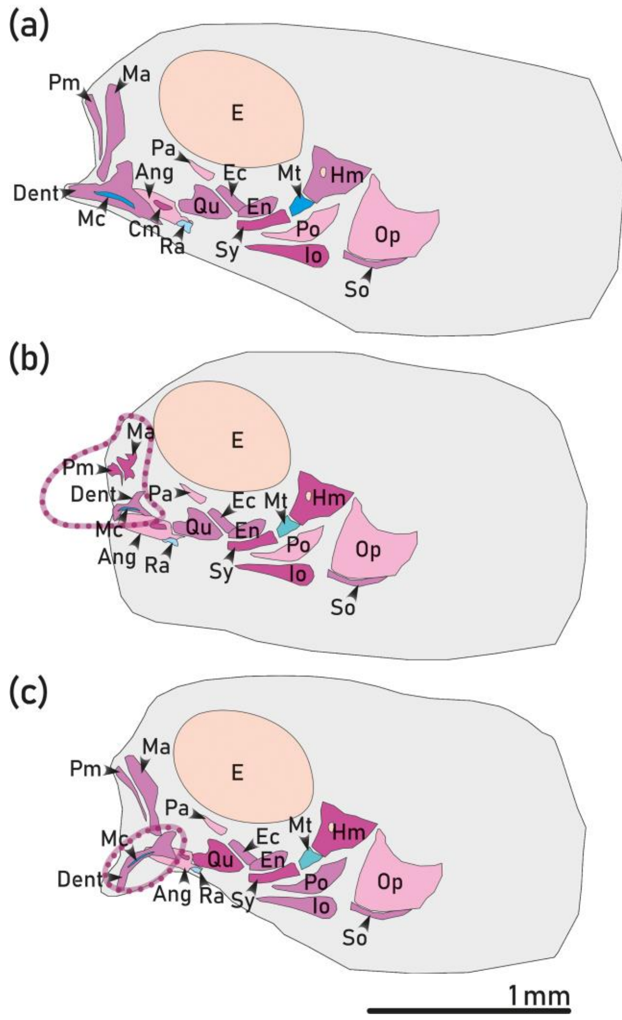

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