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ORIGINAL RESEARCH

Chromosome preparation in fish: effects of fish species and larval age

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Abstract To date, several protocols have been developed to achieve clear and identifiable metaphase chromosome spreads from larvae of a single fish species. However, the efficiency of these protocols in more than one fish species has barely been compared within a single study. This work investigated the dependency of chromosome preparation parameters including colchicine concentration (0.01, 0.025, 0.05 %) and exposure duration (3, 5 h), hypotonic solution (distilled water, 0.075 M KCl solution), and Giemsa stain solution concentration (6, 8, 10, 11, 12, and 14 %) and incubation period (15, 30, 45, and 60 min) to two species of fish, the African catfish (*Clarias gariepinus*) and the zebrafish (*Danio rerio*) at different larval ages (0, 2, and 4 days post-hatch, dph). Results indicated that larval age, colchicine concentration and/or incubation time, and/or the type of hypotonic solution varied with fish species while staining the chromosomes with 11 % Giemsa solution for 45 min can be maintained regardless of the species or larval age. Interestingly, employing the selected values from diploid *C. gariepinus* experiment to prepare metaphase chromosomes from larvae of their triploid siblings proved to be efficient.

Keywords Metaphase chromosome spread · Colchicine · Hypotonic solution · Giemsa stain · Zebrafish · African catfish

Introduction

Cytogenetic methods, such as taxonomic works, and karyological analysis are essential in providing basic information on fish breeding programs such as inter-specific hybridization (Crego-Prieto et al. 2013), chromosome manipulation techniques (Christopher et al. 2010; Gilna et al. 2014; Thresher et al. 2014), and genetic

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improvement of commercial fish stocks (Gui and Zhu 2012). To date, a variety of karyotyping techniques such as tissue cultures (Lomax et al. 2000), squashing techniques (Armstrong and Jones 2003), and cell suspensions of the tissues undergoing mitosis (Fan and Fox 1990; Henegariu et al. 2001) have been developed to visualize chromosomes of fish at different developmental stages.

Nevertheless, methods targeting embryonic and larval stages of fish have faced difficulties in achieving a reliable number of identifiable and wide-spread metaphase chromosomes probably due to the variations in mitotic cell division rates among different fish species (Shao et al. 2010). In conventional chromosome preparation protocols, a spindle poison (e.g., colchicine) is employed to arrest the cells at their metaphase stage (Kligerman and Bloom 1977). To achieve clear and identifiable metaphase chromosome spreads, it is essential to select the proper concentration and the correct incubation period of the poison (Rieder and Palazzo 1992). While insufficient concentration and/or duration of exposure to the spindle poisons fails to arrest the cells at the metaphase stage, very high concentrations and/or overly long exposure duration may lead to chromosomal condensation (Rieder and Palazzo 1992; Wood et al. 2001). Following mitotic spindle inhibition, cells or larvae must be incubated in a hypotonic solution to swell the nuclei and scatter the chromosomes on the slides (Moore and Best 2001). Choosing an improper hypotonic solution and incubation period would either make chromosomes knotted and overlapped or result in chromosomal loss (Baksi and Means 1988).

The hypotonic solution step is further followed up by fixing the samples using Carnoy's fixative solution, and preparing a cell suspension. Furthermore, during slide preparation, factors such as fixative evaporation rate and preheating of slides affect the quality and quantity of mitotic chromosomal spread (Moore and Best 2001). Therefore, the approach to slide preparation is another important step as employing improper spreading techniques would cause the chromosomes to be washed out during the staining process.

Following slide preparation, various staining techniques such as classic staining (e.g., aceto-orcein, haematoxylin, Giemsa, Wright and Leishman stains) or banding techniques (e.g., Q-banding, G-banding, R-banding, C-banding, and high-resolution banding) (Calado et al. 2013; Moore and Best 2001; Wang et al. 2010) are used to stain chromosomes for different purposes. Concentrated staining solutions and/or over-incubation would result in dark background filling the space between chromatids, whereas diluted staining solutions and/or a limited incubation period creates indistinguishable chromosomal spreads.

Table 1 provides detailed information on colchicine treatments, hypotonic solutions, fixatives, and Giemsa staining used by previous studies with fish embryos and larvae. To the best knowledge of the researchers, very limited studies have examined the requirements to alter chromosomal procedures for different species of fish and/or larval age. To fill this gap, 0, 2, and 4 days post- hatch (dph) zebrafish (*Danio rerio*), a widely used vertebrate model, and African catfish (*Clarias gariepinus*), a widespread tropical/sub-tropical species, were used and the results compared. A stepwise procedure was followed to determine the key elements in obtaining the highest number of mitotic chromosome spreads pertaining to the species of fish and larval age.

Materials and methods

Chemicals

Colchicine, methanol (99 % HP grade), glacial acetic acid, and KCl were purchased from R&M chemicals (UK), and Giemsa stain from Merck. Distilled water (HPLC grade) was produced daily in the laboratory.

Fish

Immature African catfish *C. gariepinus* and wild-type *D. rerio* were purchased from local suppliers and reared for 3 months at 28 °C on a 12:12 dark/light photoperiod in a recirculation system, and fed three times daily. 0, 2, and 4 days post-hatch (dph) larvae of *C. gariepinus* and *D. rerio* were obtained according to Karami et al. (2010) and Nasiadka and Clark (2012), respectively. Triploid *C. gariepinus* were produced as described by Karami et al. (2011).

Table 1 Comparison of chromosome preparation methods using fish embryos and larvae

Fish species	Developmental stage	Colchicine	Hypotonic solution: incubation period (min)	Fixation method	Giemsa concentration (%), incubation period (min)	References
Cyprinodon variegatus and *Morone saxatilis*	Embryo and 0 dph larvae	0.05 % for 4 h	Distilled water: 90	Carnoy's fixative	10, 20	Baksi and Means (1988)
Acipenser baeri × *Acipenser gueldenstaedti*	0 dph larvae	0.025 % for 4 h	KCl (0.075 M): 60	Carnoy's fixative	5, 20	Fopp-Bayat and Woznicki (2006)
Oreochromis niloticus, *Slamo salar*, and *Salmo trutta*	0 dph larvae	0.002–0.005 % for 4–6 h	Distilled water: 8–12	4:1 methanol acetic acid	10, 10–20	Hussain and McAndrew (1994)
Oncorhynchus mykiss, Oncorhynchus musou, Oncorhynchus keta	Embryos	0.0012–0.0025 % for 6–12 h	0.8 % tri-sodium citrate dihydrate: 15–30	Carnoy's fixative	4, 30	Inokuchi et al. (1994)
Clarias gariepinus	0 dph larvae	0.05 % for 3 h	Distilled water: 30	Carnoy's fixative	14, 45	Karami et al. (2010)
Oreochromis mossambicus × *O. niloticus*	0 dph larvae	0.01 % for 4–6 h	KCl (0.075 M): 40	Carnoy's fixative	10, 20	Pradeep et al. (2011)
Cynoglossus semilaevis	Embryos and 0 dph larvae	0.02 % for 1–2 h	KCl (0.075 M): 30	Carnoy's fixative	10, 10	Shao et al. (2010)

dph days post hatch

Chromosomal preparation procedure

In this study, the method of Shao et al. (2010; Table 1), which is a modified method of Kligerman and Bloom (1977), was used as the basis for chromosomal preparation. Accordingly, all the variables have been optimized in a stepwise manner. In all stages, five larvae were used for each treatment and two slides were prepared from each larva. Larvae were killed by smashing the head with forceps and the yolk sac was removed carefully prior to starting the experiments (Thorgaard et al. 1981).

Colchicine

Larvae were incubated in 0.01, 0.025, or 0.05 % colchicine for 3 or 5 h. The procedure was followed as described by Shao et al. 2010; Table 1). Finally, on each slide, clear metaphase chromosome spreads were counted. In all stages, the metaphase spreads were photographed under 1000 X (oil immersion) using a Motic BA400 microscope.

Hypotonic solution

Using the selected values of colchicine concentration and incubation period, colchicine-treated larvae were incubated in distilled water (Karami et al. 2010) or 0.075 M KCl solution (Pradeep et al. 2011) for 30 min, followed by the procedure of Shao et al. (2010; Table 1). Finally, clear chromosome spreads were counted on each slide.

Fixation

Following the optimization of colchicine concentration and incubation period, and hypotonic solution for the different larval age in each species, larvae were fixed in chilled Carnoy's fixative solution (ethanol:glacial acetic acid 3:1 vol/vol) for a total of 40 min. The solution was renewed every 20 min. No alternative fixative solution was considered as Carnoy's solution is the most widely used fixative found in the previous studies (Fopp-Bayat and Woznicki 2006; Karami et al. 2010; Pradeep et al. 2011, Table 1).

Slide preparation method

Each *C. gariepinus* and *D. rerio* larva was minced gently in 40 and 20 µl of 50 % acetic acid, respectively. Afterwards, 10 µl of the cell suspension was pipetted onto a ethanol-cleaned slide. Slide were held 3–5 cm above the flame while the droplet was continuously trundled on the slide in a dilatory spiral movement making a 0.75- to 1-cm diameter circle (Fig. 1).

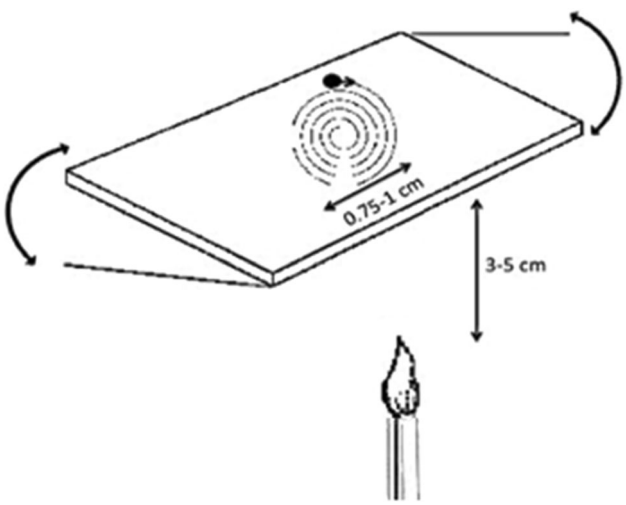

Fig. 1 Spiral movement of dropped suspension on the slide during flame drying

Giemsa stain

Slides were incubated in freshly prepared 6, 8, 10, 11, 12, or 14 % Giemsa solution for 15, 30, 45, or 60 min. The optimum concentration and duration of Giemsa stain were chosen based on the visibility of chromosomes, background clarity, and distinction between chromatids within a single chromosome.

Due to the wide application of chromosomal preparation technique in ploidy determination, the optimized protocol for 2 dph *C. garipinus* larvae was tested on 2 dph triploid *C. garipinus*.

Statistical analyses

To improve normality and homogeneity of variance, chromosome spread counts were arcsin-square-root transformed. Prior to running ANOVA analyses, normality and homogeneity of data had been tested (Tabachnick and Fidell 2001). A three-way ANOVA was employed to evaluate the interactions and effects of colchicine concentration, exposure duration, and larval age in *C. gariepinus* and *D. rerio*. A two-way ANOVA was applied to elucidate the interactions and effects of hypotonic treatments and larval age. The ANOVA tests were followed by post hoc Duncan's multiple range tests when significant differences ($P < 0.05$) were detected. All statistical comparisons were performed through IBM SPSS Statistics Version 21.

Ethics statement

Animal ethics approval was obtained from the Animal Ethics Committee, UPM (reference no: UPM/IACUC/AUP- R076/2013).

Results

Throughout the study, 4 dph larvae failed to produce countable mitotic chromosome spreads in both *C. gariepinus* and *D. rerio*; therefore, they were excluded from the statistical analyses. Colchicine concentration and duration, hypotonic solution, cell suspension density, Giemsa stain concentration and duration were optimized according to the following results:

Colchicine

African catfish

Except for one interaction (larval age × colchicine incubation period), the interactions among colchicine concentration, incubation period, and larval age on the number of chromosome spreads were non-significant ($P > 0.05$). The effect of larval age on the number of metaphase chromosome spreads differed with colchicine incubation period (Three-way ANOVA, $F_{1,24} = 18.28$, $P < 0.01$). In 2 dph larvae, 3 h of incubation in colchicine provided significantly higher number of chromosome spreads; however, the incubation period did not significantly alter the number of chromosome spreads in 0 dph larvae. Figure 2a depicts changes in the number of metaphase chromosome spreads within different colchicine concentrations. Figure 2b shows the number of metaphase chromosome spreads of 0 and 2 dph larvae following 3- and 5-h incubation in colchicine solution.

Zebrafish

The interactions between larval age × colchicine concentration (Three-way ANOVA, $F_{1,24} = 3.37$) and larval age × colchicine incubation period (Three-way ANOVA, $F_{1,24} = 14.37$) proved to be significant ($P < 0.05$). Figure 2c, d displays changes in the number of metaphase chromosome spreads within different colchicine concentrations and colchicine incubation time, respectively. The results from a three-way ANOVA failed to show significant interaction between the effects of colchicine concentration, exposure duration, and larval age on the number of chromosome spreads.

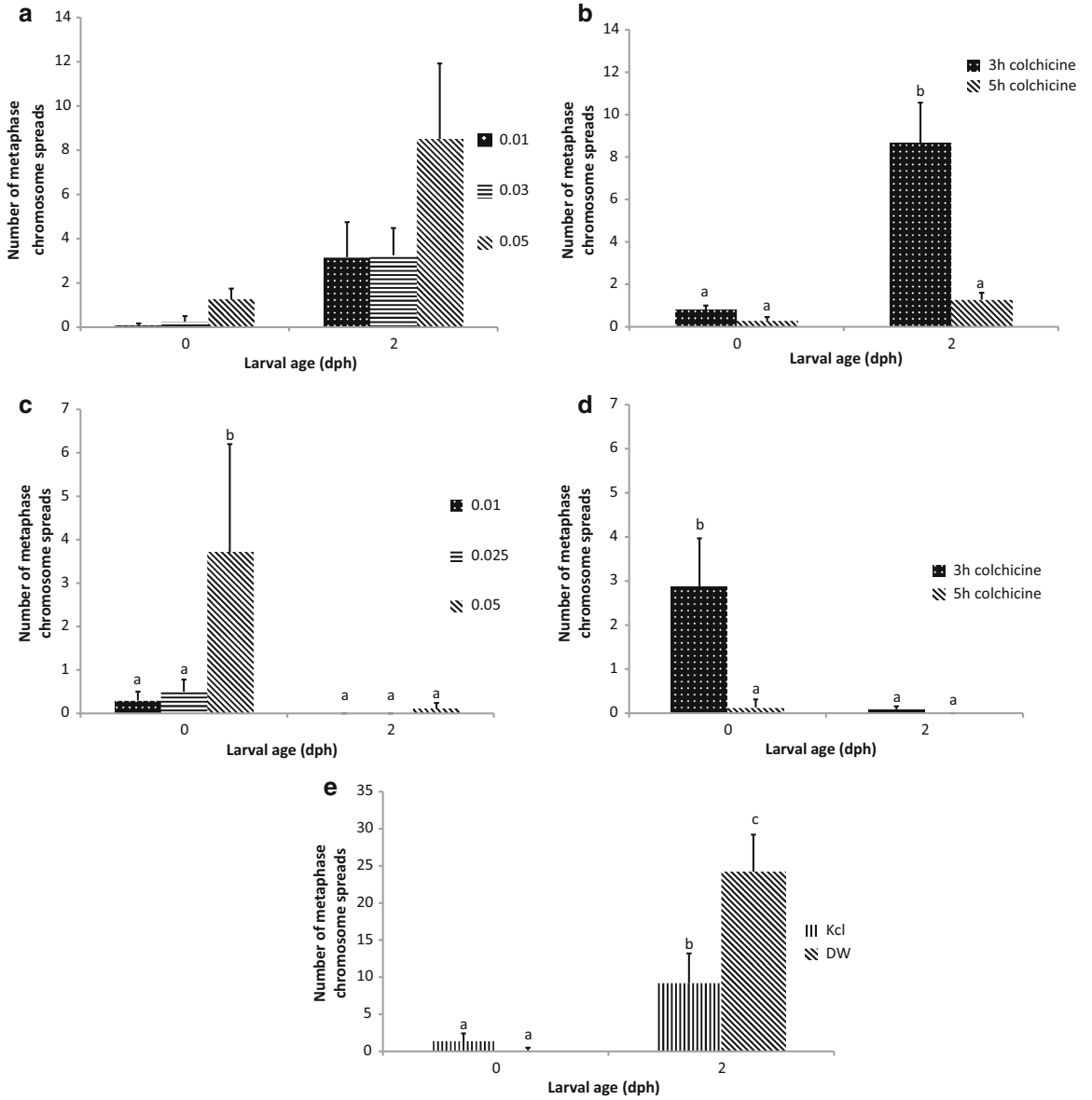

Fig. 2 Effects of different treatments on the number of clear and identifiable metaphase chromosome spreads **a** larval age × colchicine concentration interaction in *C. gariepinus*, **b** larval age × colchicine incubation period interaction in *C. gariepinus*, **c** larval age × colchicine concentration interaction in *D. rerio*, **d** larval age × colchicine incubation period interaction in *D. rerio*, **e** hypotonic treatments in *C. gariepinus*. The data shown here are Mean + SE. *Bars* noted by *different letters* are significantly different from each other ($P < 0.05$, Duncan's multiple range test)

Hypotonic solution

African catfish

The interaction between hypotonic treatment and larval age on the number of metaphase chromosome spreads was significant (Two-way ANOVA, $F = 8.65$, $P < 0.05$). Duncan's multiple range tests proved that for 0 dph *C. gariepinus*, there was no significant difference in using distilled water or KCl solution (Fig. 2e). However, using distilled water would significantly increase the number of metaphase chromosome spreads in 2 dph larvae (Fig. 2e).

Zebrafish

The result of this experiment showed the types of hypotonic solution do not have significant interactions with larval age to augment the number of metaphase chromosome spreads in zebrafish.

Giemsa staining

Regardless of the duration of incubation, using a Giemsa stain at concentrations lower than 11 % produced non-visible chromosomes in all the treatments. In contrast, using 12 % and particularly 14 % Giemsa caused sedimentation, dark background and filled the space between chromatids. In general, in both species desirable results were achieved when the spreads were stained with 11 % Giemsa for 45 min making chromosome spreads suitable for karyotyping (Fig. 3a–c).

Discussion

Most chromosomal preparation protocols for fish have been designed for a single species. To the best knowledge of the researchers, to date, no study has evaluated the modification of these protocols according to the fish species and larval age. For the first time, this study proved the necessity of modifying colchicine concentration and incubation period, and the type of hypotonic solution according to the species of fish and the larval age.

Obtaining a desirable number of mitotic chromosome spread is directly related to the number of cells undergoing mitotic division. In turn, mitotic rate is a factor influenced by species and environmental parameters (Shelton et al. 1997). By comparing metaphase chromosome numbers and mitotic rates in different life stages of *Cynoglossus semilaevis*, Shao et al. (2010) found a higher mitotic rate in the larvae compared to the juveniles and adults. In a similar way, differences in mitotic division rates may explain why in this study different aged *C. gariepinus* and *D. rerio* larvae responded differently to the colchicine concentration and duration, and to the hypotonic solutions. In another study, Wakahara (1972) found a higher mitotic rate in the ventral tail-fin epidermis of the larval African clawed frog (*Xenopus laevis*) during the night time compared to the day time, thus highlighting the importance of environmental factors in getting a reliable number of chromosome spreads.

Chourrout and Happe (1986) pinpointed the necessity of using less than 170-day-old larvae of *Salmo gairdneri* to achieve a high number of metaphase chromosomes. In the current study, the highest number of metaphase chromosome spread was recorded in 0 dph *D. rerio* and 2 dph *C. gariepinus*. Therefore, different aged larvae may be used when different fish species are involved.

Colchicine is the most common microtubular poison used (Table 1). It has been reported that before nuclear envelope breakdown (NEB) in the metaphase cells, sufficient concentration of colchicine inhibits spindle microtubules and disperses the metaphase chromosomes in the cytoplasm (Caperta et al. 2006), whereas in *D. rerio* both colchicine concentration and incubation period were significantly influenced by larval age, and in *C. gariepinus* only colchicine incubation period was dependent on the larval age. The sensitivity of microtubule polymers to physical and chemical parameters has been shown before (Tilney and Porter 1967; Weisenberg 1972). Therefore, age- and species-dependent cellular parameters may influence the sensitivity of cells towards depolymerizing effects of colchicine. According to the results of this study, to achieve clear and identifiable metaphase chromosome spreads, colchicine concentration and/or incubation period should be altered according to the fish species and/or larval age.

Using an appropriate hypotonic solution is the other critical element that has been emphasized in the current study. Potassium chloride (KCl 0.075 M) is one of the most commonly used hypotonic solutions in chromosomal preparation protocols (Table 1). Analogously, the efficacy of distilled water as a hypotonic treatment has been shown in some other protocols (Table 1). In *C. gariepinus*, the number of clear metaphase chromosome spreads was significantly increased when distilled water was used compared to KCl. Using KCl caused extensive cell burst and chromosomal loss. In contrast, in *D. rerio* the changes in the hypotonic solution did not significantly influence the number of metaphase chromosome spreads. This study showed that

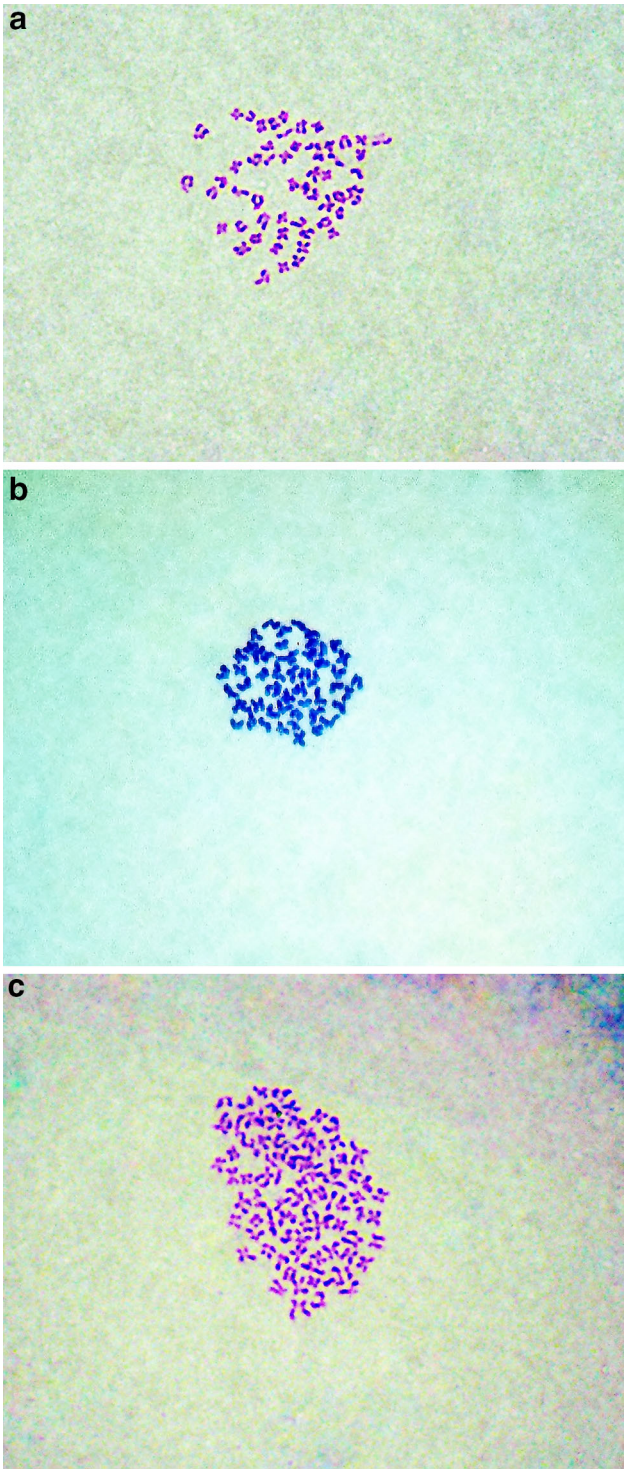

Fig. 3 Metaphase chromosome spreads of **a** diploid *C. gariepinus* ($2n = 56$); **b** diploid *D. rerio* ($2n = 50$); and **c** triplioid *C. gariepinus* ($n = 84$)

to procure a desirable number of clear chromosome spreads, the type of hypotonic solution should be modified according to the species of fish and/or larval age.

Besides the aforementioned elements, this study attempted to modify other important factors of chromosomal preparation protocols. Our preliminary studies proved that the larvae should be killed prior to the incubation in colchicine as incubating live larvae in colchicine solution failed to produce chromosome spread. Furthermore, to obtain clear chromosome spread, the yolk sac should be removed prior to the incubation in

colchicine (Hussain and McAndrew 1994; Pradeep et al. 2011) because high lipophilicity of the yolk may restrict the penetration of colchicine or hypotonic solution to the cells (Baksi and Means 1988).

The quality of slides is affected by the proper staining solution concentration and the duration of incubation. This study showed that using 11 % of Giemsa solution for 45 min could lead to better quality of stained chromosomes in both fish species and in the polyploid fish. Most of the previous methods employed concentrations similar to this study but with a lower incubation period (e.g., Hussain and McAndrew 1994; Pradeep et al. 2011; Shao et al. 2010). Some other studies used lower concentrations such as 4–5 % (Fopp-Bayat and Woznicki 2006; Inokuchi et al. 1994; Kligerman and Bloom 1977) or higher concentrations like 14 % (Karami et al. 2010). The results of this study showed that concentrations less than 11 % and incubation period below 45 min would lead to unclear chromosomes. In contrast, higher concentrations encouraged sedimentation; therefore, dark background was achieved and the spaces between chromatids were stained. Concentration and incubation period were neither affected by larval age nor fish species; therefore, incubating the slides at 11 % of Giemsa for 45 min could be extended to other fish species. Despite having the chromosome preparation parameters optimized for the two species, several specimens of each species did not produce well-spread metaphase chromosomes, which are likely due to the interference of physiological factors. Further studies are required to understand the role of these elements in cytogenetic studies.

This study proved that depending on the fish species and the age of larvae, colchicine concentration and/or exposure duration, and/or hypotonic solution must be altered to achieve clear metaphase chromosome spread. Furthermore, the Giemsa stain concentration and incubation period proved to be independent of species and larval age. Probably significant differences on the amount of yolk content and its rate of absorption, and the differences on the rate of cell division among larvae of different fish species are responsible for differences among chromosome preparation protocols. These factors are either absent or less effective when juvenile or adult fish are of concern.

Author's contributions AK and PEA formulated the hypothesis and designed the study. AK conducted the experiments. AK and PEA analyzed the data. AK wrote the manuscript with assistance from SPW and MAS. All authors read and approved the final manuscript.

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Conflict of interest The authors declare that they have no conflict of interests.

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