

Short communication. Zebrafish embryo development can be reversibly arrested at the MBT stage by exposure to a water temperature of 16°C

M. Francisco Simão^{1,2*}, M. Pérez Camps¹ and F. García-Ximénez¹

¹ *Laboratory of Animal Reproduction and Biotechnology. Polytechnic University of Valencia (LARB-UPV). Camino de Vera, 14. 46071 Valencia. Spain*

² *Faculty of Agricultural Sciences. Agostinho Neto University. Angola*

Abstract

Germline chimaerism (intra or interspecific) is a technique of great potential in aquaculture. It allows specimens to be obtained that produce gametes whose origins lie in the cells of the donor organism. Chimaerism is usually performed at the mid blastula transition (MBT) stage since this is the last in which embryonic cells remain completely totipotent. Zebrafish are photoperiodic in their egg-laying behaviour and show rapid embryonic development. For chimaerism to be successful, it is of interest to establish the maximum time over which embryonic development can be reversibly arrested. This paper reports the effect on survival of subjecting zebrafish embryos at different stages of development to a water temperature of 16°C for different lengths of time. The maximum exposure time after which these embryos were able to resume development following low-temperature-induced developmental arrest became shorter as the embryonic stage exposed became earlier. At the MBT stage, the maximum safe exposure time was 2 h; longer exposure times led to problems in development and survival.

Additional key words: *Brachydanio rerio*, chimaerism, embryo culture, low temperature.

Resumen

Comunicación corta. El desarrollo de embriones de pez cebra en estadio MBT puede ser detenido reversiblemente a 16°C

El quimerismo intra e interespecífico en la línea germinal es una técnica con un gran potencial para la Acuicultura, ya que permite obtener especímenes en los que algunos gametos provienen de células (de la misma o de diferentes especies de interés) insertadas en el embrión. El quimerismo se efectúa habitualmente en el estadio MBT (*mid blastula transition*), ya que éste es el último estadio en el que las células embrionarias aún son totipotentes. El pez cebra es una especie de reproducción fotoperiódica y de rápido desarrollo embrionario, lo que hace de interés evaluar el tiempo y el estadio preciso en que es posible detener reversiblemente el desarrollo sin que éste resulte penalizado posteriormente. Se ha evaluado el efecto que ejerce la permanencia a 16°C sobre la supervivencia de embriones de pez cebra en diferentes estadios. El tiempo máximo de exposición tras el cual los embriones pueden reanudar su desarrollo es tanto más limitado cuanto más temprano es su estadio. En el caso concreto de embriones de pez cebra en estadio MBT, éstos pueden ser mantenidos a 16°C durante 2 h como máximo sin penalizar su posterior supervivencia y desarrollo.

Palabras clave adicionales: baja temperatura, *Brachydanio rerio*, cultivo de embriones, quimerismo.

In aquaculture, somatic chimaerism (intra- or inter-specific) is an important technique used to produce fish that show characteristics possessed by both the recipient and donor organisms (Nakagawa and Ueno, 2003; Sawant *et al.*, 2004). Germline chimaerism allows specimens to be obtained in which the origin of

the gametes lies in the cells of both participants (Fan *et al.*, 2004). This is a powerful technique that can be used to help obtain transgenic animals (Fan and Collodi, 2002).

Zebrafish (*Brachydanio rerio*), which are photoperiodic in their egg-laying behaviour and produce eggs every morning at dawn (Westerfield, 1993), as well as other fish species showing rapid embryonic development, are widely used as animal models in

* Corresponding author: fransim8@yahoo.com.br
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biotechnological and aquacultural research. This rapid development, however, hinders the successful undertaking of lengthy procedures such as chimaerism which can only be performed at certain, rapidly-passing stages of development. Knowing how to suspend embryonic development at the mid blastula transition (MBT) stage, when chimaerism is usually performed (Lin *et al.*, 1992; Kane and Kishimoto, 2002; Nakagawa *et al.*, 2002), or even in earlier developmental stages, would therefore be very advantageous.

It is well known that a reduction in water temperature can delay embryonic development. Its effects on embryo survival, hatching ability and the presence of abnormalities have been studied in a number of fish species (Gray, 1928; Rechulicz, 2001; Arenzon *et al.*, 2002; Ojanguren and Braña, 2003). However, all these effects were tested over temperature ranges that did not involve developmental arrest. The aims of the present work were to determine embryonic resistance to low temperatures in order to try to arrest development completely, and to determine for how long, and under what conditions, early zebrafish embryos can withstand developmental arrest without suffering negative consequences upon its resumption. The literature appears to contain no information on this.

Embryos were collected by siphoning them from an aquarium containing adult members of a wild zebrafish colony (established three years ago in our laboratory; maintained with a 2:1 female:male ratio). Granular food (Tetra GMBH: 46% proteins, 2% fibre, 5% fat, 6% humidity) was supplemented with recently defrosted chicken egg yolk and shrimp meat in substitution of live food as recommended for egg production in zebrafish (Goolish *et al.*, 1999). All chemical products and culture media were purchased from Sigma-Aldrich.

After washing the embryos on a nylon mesh with conventional chlorinated water, those at the MBT stage



Figure 1. MBT embryo dechoriation by pronase treatment. A: with chorion (1.1-1.2 mm of diameter). B: without chorion (0.7-0.8 mm of diameter).

were selected under a stereomicroscope and washed again with chlorinated water.

A sample of the embryos were dechorionated by pronase treatment (1.5 mg ml⁻¹ in 10-H, i.e., 10% Hanks' balanced salt solution; Westerfield, 1993) followed by immersion twice in 10-H (Fig. 1). Partially or totally damaged embryos were removed. No bleaching treatment was performed. Strictly sterile media and Pasteur pipettes were used after dechoriation.

Two preliminary assays were performed in 10-H embryo medium, the first to determine the threshold (descending) temperature at which non-dechorionated embryo development can be arrested yet which allows it to be resumed. The embryos at different stages continued their development in a limited manner when maintained at > 17°C for 4 h. Although cell division was slower, no complete, immediate arrest of development was observed. Thus, 16°C was established as the threshold temperature to be used in the following assays.

The second preliminary assay attempted to establish the maximum length of time that non-dechorionated embryos at different stages of development could withstand remaining at 16°C yet maintain their ability to resume development. The ability of epiboly stage embryos to resume development after long periods (up to 24 h) of suspension was also tested (Table 1). Embryos at 50% epiboly could withstand 16°C for 4 h. Similar

Table 1. Preliminary assay 2: ability of non-dechorionated epiboly stage embryos to resume normal development after 4 h and 24 h at 16°C

Embryonic stage	After 4 h at 16°C		After 24 h at 16°C	
	Initial embryos	Larvae obtained (hatching stage)	Initial embryos	Larvae obtained (hatching stage)
50% epiboly	47	47	—	—
75-100% epiboly	31	30	47	47 ¹

¹ All larvae obtained were morphologically very abnormal.

Table 2. Survival rates of embryos cultured at 16°C for 4 h depending on their developmental stage and whether they underwent dechoriation

	Early MBT (B-D stage)	MBT (E-F stage)	Late MBT (G-J stage)
Dechoriated (in 10-H)	0/11 (0%) ^a	8/20 (40%) ^b	37/38 (97%) ^c
Non-dechoriated (in deionised water)	0/14 (0%) ^a	91/172 (53%) ^b	76/76 (100%) ^c
Total	0/25 (0%) ^a	99/192 (52%) ^b	113/114 (99%) ^c

Figures with different letters are statistically different ($p < 0.05$).

results were obtained when 75-100% epiboly embryos were used. In fact, these embryos were able to resist for up to 24 h at this temperature although they showed important abnormalities at hatching (enlargement of the pericardium and altered body morphology), or even an inability to hatch.

Two experiments were then performed involving 20 dechoriated and 20 non-dechoriated embryos at the MBT stage or earlier (during which they are very sensitive to low temperatures). At least three replicates were prepared for all experimental groups. The results were analysed using the Chi-square test. When a single degree of freedom was involved, Yates correction for continuity was performed. In the first experiment, the effect on survival of subjecting dechoriated and non-dechoriated early MBT (B to D), MBT (E to F) and late MBT (G to J) (Kimmel *et al.*, 1995) embryos to a water temperature of 16°C for 4 h in 10-H was determined. Table 2 shows that the embryos suffered limitations to their development when the exposure time went beyond 4 h. This was more evident the earlier the embryonic stage exposed. Dechoriation appeared not to influence the results obtained.

Early MBT is the usual stage for undertaking chimaerism. Blastomeres extracted from MBT embryos

become lysed immediately when they are placed in embryo medium due to its low osmolarity (10-H: 35 mOsm). However, embryos can be kept in cell medium (Complete Hanks' – CH: 315 mOsm) for a short time with no adverse osmolarity effects (unpublished). Therefore, in experiment 2, the same low temperature treatment was undertaken in 10-H (35 mOsm) or CH (315 mOsm). Non-dechoriated embryos were exposed to 16°C for 0 h (control), 1 h, 2 h or 3 h. After the temperature treatment they were all dechoriated and maintained in 10-H. Survival rates at 24 h under normal conditions were then evaluated (Table 3). The time that the embryos were kept at 16°C significantly affected their survival rate. The lowest survival rate was obtained after a 3 h exposure to 16°C, although no significant differences were recorded except when compared with the control group. The results were similar for both the 10-H and CH media conditions. Thus, thermal treatment to arrest development before performing chimaerism should be performed in CH since it is in this medium that cells have to be transplanted into MBT embryos.

Since both organogenesis and somatic growth are controlled by enzymes, the embryonic development of ectotherms depends on the differential expression of certain genes and on temperature (Ojanguren and Braña,

Table 3. Survival rates of MBT embryos after 16°C treatment for different exposure times (0, 1, 2, 3 h) in the culture media CH (complete Hanks') or 10-H (10% Hanks'), evaluated after dechoriation and at 24 h of culture

	Number of non-lysed embryos (1-2 h after dechoriation)			Number of larvae (24 h after 16°C treatment)		
	CH	10-H	Total ¹	CH	10-H	Total ¹
1 h	43/49 (88%)	35/49 (71%)	78/98 (80%) ^a	34/43 (79%)	32/35 (91%)	66/78 (85%) ^{ab}
2 h	36/39 (92%)	38/39 (97%)	74/78 (95%) ^b	33/36 (92%)	28/38 (74%)	61/74 (82%) ^{ab}
3 h	23/29 (79%)	26/29 (90%)	49/58 (84%) ^{ab}	16/23 (70%)	18/26 (69%)	34/49 (69%) ^b
Control	35/44 (80%)	36/44 (82%)	71/88 (81%) ^a	28/35 (80%)	34/36 (94%)	62/71 (87%) ^a
Total	137/161 (85%)	135/161 (84%)	272/322 (85%)	111/137 (81%)	112/135 (83%)	223/272 (82%)

¹ Figures with different letters are statistically different ($p < 0.05$).

2003). If the incubation temperature is reduced, the speed of embryonic development diminishes, although the range of temperatures over which healthy, functional larvae can be produced is wide in most - if not all - fish species (Gray, 1928). In the present preliminary work, the incubation of MBT embryos at 17°C did not immediately halt development, although the rate was much lower than at the optimum 28.5°C (Westerfield, 1993). The effect of low temperature seems to be greater the earlier the developmental stage exposed. In fact, the exposure of recently fertilized zebrafish embryos to 15°C prevents them developing beyond the 4-cell stage; if they are exposed at 18°C they only reach the 8-cell stage (Schirone and Gross, 1968).

Diapause is a natural phenomenon very common in annual fish species, whereby eggs undergo developmental arrest during unfavourable climatic periods (Arenzon *et al.*, 2002). The artificial induction of diapause is a normal commercial practice in poultry production; it may even last several days. In fact, the arrest of embryonic development in mammals by cryopreservation is also habitual. The latter technique, however, is associated with great difficulties in fish (Chen and Tian, 2005).

The present results show that zebrafish development can only be arrested during its early stages for a few hours. It should be noted that the maximum time that can be spent at 16°C without negative effects occurring becomes shorter the earlier the developmental stage exposed. For early MBT stage embryos this should be no longer than 2 h, whereas epiboly stage embryos can last 24 h (although the rate of morphological anomalies increases, survival seems unaffected). This suggests that thermal limits are narrower for early stage embryos, in which organogenesis predominates over growth. This agrees with the almost general principle that earlier developmental stages are more stenothermal (Elliot, 1981; Cossins and Bowler, 1987).

Finally, the reduced hatchability detected in the second preliminary study might be related to the dynamics of the hatching gland cells (HGCs). The appearance of the HGCs is closely related to the moment at which the eye pigment appears and when the heart begins to beat and blood circulation begins. These cells appear earlier in eggs incubated at high temperatures and take longer to appear in those incubated at lower temperatures (Rechulicz, 2001). This might be important in commercial low-temperature transport of epiboly stage embryos; these will require mechanical or enzyme assistance if they are to hatch.

In conclusion, MBT zebrafish embryos can be maintained at 16°C for 2 h without risk to their subsequent survival and development, in order to facilitate chimaerism. Embryos could be subjected to this treatment before dechoriation and in a medium that has no adverse osmolarity effects on donor cells.

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