Journal of Thermal Biology 74 (2018) 170–173

Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: www.elsevier.com/locate/jtherbio

Larval rearing of zebrafish at suboptimal temperatures

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ARTICLE INFO

Keywords:
Temperature-sensitive
Conditional mutant
Zebrafish
Screen
Husbandry

ABSTRACT

Temperature-sensitive mutants have been widely utilized in single-cell and invertebrate model systems, particularly to study the function of essential genes. Few temperature-sensitive mutants have been identified in zebrafish, likely due to the difficulty of raising zebrafish at low temperatures. We describe a novel rearing protocol that allows rapid growth of larval and juvenile zebrafish at 23 °C compared to previous data in the literature. Embryos collected from four breeding pairs were maintained at 28.5 ± 0.5 °C until 5 days post-fertilization (dpf) – the onset of exogenous feeding. Larvae were then divided to six tanks and three tanks were cooled to 23 ± 0.2 °C. Fish were fed a live diet (marine rotifers *Brachionus plicatilis* and *Artemia* nauplii) and maintained under a set of environmental parameters shown to increase growth rate: continuous light, low salinity (3ppt), and algal turbidity. Mean total length and weight of fish at 21dpf were 12.7 ± 0.3 mm and 20.5 ± 1.5 mg for the 23 °C treatment and 18.5 ± 0.4 mm and 67.3 ± 3.4 mg for the 28.5 °C control. By 35 dpf, the fish raised at 23 °C had reached a mean length and weight of 18.9 ± 0.7 mm and 76.4 ± 6.7 mg, approximately the size control fish reached at 21 dpf. At 35 dpf, water temperature was raised to 28 °C and fish were reared to maturity (75 dpf) under standard conditions (freshwater, 13 L:11D photoperiod, dry diet, no added algal turbidity). Sex ratio and fertility were assessed and compared between temperature groups. There were no significant differences in sex ratio, fertilization rate, embryo viability at 1 dpf, clutch size, or relative fecundity. This rearing protocol will allow for efficient utilization of temperature-sensitive mutations in the zebrafish model system.

1. Introduction

Zebrafish were the first vertebrate species to be used in a large-scale genetic screen. These screens identified loss-of-function mutants for multiple genes involved in embryonic development (Driever et al., 1996; Haffter et al., 1996), and success has continued with screens for complex traits, such as behavioral phenotypes (Chiu et al., 2016). The functions of many essential genes are difficult to analyze in loss-of-function mutants due to mortality of the embryo. In other model systems, such as yeast, this has been overcome by screening for conditional mutants, in which the mutated gene is functional under one set of environmental conditions but not fully functional under other conditions.

The most frequent examples of conditional mutants are temperature-sensitive mutants. Screens for temperature-sensitive yeast mutants have most notably identified essential genes involved in cell-cycle control (Hartwell et al., 1970; Nurse et al., 1976) and currently there are temperature-sensitive mutant strains available for close to half of all essential genes in *Saccharomyces cerevisiae* (Li et al., 2011). Temperature-sensitive mutants have also been used to study development throughout the life cycle and cell-cycle control in *Caenorhabditis elegans* (Hirsh and Vanderslice, 1976; O'Connell et al., 1998).

Zebrafish can tolerate (based on loss of equilibrium) water temperatures as low as 6.2 and 10.6 °C when acclimated to 20 and 30 °C, respectively (Cortemeglia and Beitinger, 2005) which allows temperature shifts to be used as an experimental tool in this vertebrate model system (López-Olmeda and Sánchez-Vázquez, 2011). A small number of temperature-sensitive mutants have been found in zebrafish. Temperature-sensitive mutations affecting fin regeneration have been identified (Johnson and Weston, 1995; Nechiporuk et al., 2003) with a permissive temperature of 25 °C, slightly below the optimum temperature for growth of zebrafish (28 °C), and a restrictive temperature of 33 °C. Additionally, one temperature-sensitive mutation in *nodal-related 2* (*ndr2*), also known as *cyclops*, a nodal-related signaling factor involved in floor-plate specification, was found (Tian et al., 2003). This allowed precise determination of the timing of *ndr2* action during embryonic development through temperature shift experiments. Temperature shift experiments were not performed on juvenile fish to investigate the function of *ndr2* after the completion of embryonic development, likely due to the difficulty of raising zebrafish at the permissive temperature (22 °C).

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https://doi.org/10.1016/j.jtherbio.2018.03.017
Received 8 February 2018; Received in revised form 2 March 2018; Accepted 18 March 2018
Available online 19 March 2018
0306-4565/

Table 1
Survival and growth of fish raised at standard (control) and low temperatures during larval and early juvenile stages. All values are given as tank mean ± SD. *calculated with an initial weight of 0.25 mg based on bulk weighing of 25 larvae at 5 days post-fertilization (dpf).

Age (dpf)	21		35	62		75	
Temperature group	Low	Control	Low	Control		Low	
Sex	Juvenile			Male	Female	Male	Female
Survival (%)	86.1 ± 2.7	90.0 ± 3.0	85.8 ± 2.9	86.9 ± 2.8		82.1 ± 6.2	
Weight (mg)	20.5 ± 1.5	67.3 ± 3.4	76.4 ± 6.7	428 ± 10	589 ± 74	434 ± 18	572 ± 46
Length (mm)	12.7 ± 0.3	18.5 ± 0.4	18.9 ± 0.7	36.4 ± 0.4	38.4 ± 1.7	36.7 ± 0.4	37.8 ± 0.8
SGR (% day^{-1})	27.5 ± 0.5*	35.0 ± 0.3*	9.4 ± 0.5	4.5 ± 0.1	5.3 ± 0.3	4.4 ± 0.1	5.0 ± 0.3

Unlike yeast, there has not been widespread screening for temperature-sensitive mutations of essential genes in zebrafish. This is presumably due to the difficulty of raising zebrafish at low temperatures. Even at optimum temperatures, there is wide variation between laboratories in growth and survival of zebrafish (Dabrowski and Miller, 2018; Lawrence, 2011).

Recently, a new method of rearing zebrafish that results in high survival and faster growth compared to traditional protocols was developed (Dabrowski and Miller, 2018; Delomas and Dabrowski, 2018). This method utilizes continuous access to live food, low salinity, algal turbidity, and continuous light during the larval and early juvenile stages to increase growth rate. The low salinity allows marine food organisms to stay alive for 24 – 48 h (Conte et al., 1972; Walker, 1981), continuous light allows continuous foraging, as zebrafish do not forage in the dark (Carrillo and McHenry, 2016; McElligott and O'malley, 2005), and the algal turbidity increases contrast of the live food organisms, decreases aggressive interactions between larvae, and acts as a food source for the marine live food (McEntire et al., 2015; Naas et al., 1992; Reitan et al., 1997; Rieger and Summerfelt, 1997).

We assessed whether (1) this novel rearing method could be applied at temperatures close to the minimum for growth, (2) result in high survival (3) acceptable growth rate and metamorphosis to juveniles, and (4) sexual maturation after returning to optimum temperatures.

2. Materials and methods

2.1. Broodstock care and reproduction

Broodstock zebrafish were from an AB/TL hybrid line and were kept in a freshwater recirculating system (Thoren Aquatic Systems, Hazleton, PA, USA) maintained at 28 ± 1 °C with a 13 L:11D (light: dark) photoperiod. Fish were fed *Artemia* nauplii supplemented with dry feed (Otohime B2, Reed Mariculture, Campbell, CA, USA). Four breeding pairs were naturally spawned on the same day. Pairs were placed in a tank with a perforated false bottom overnight and allowed to spawn naturally the following morning. Oocytes were collected from all four pairs and incubated in mesh baskets suspended in a recirculating system (Pentair Aquatic Ecosystems, Apopka, FL) maintained at 28.5 ± 0.5 °C. At 5 days post-fertilization (dpf), swim-up larvae were collected and combined. Larvae were distributed randomly into six static water tanks with a water volume of 4 L and 120 larvae per tank (30 fish /L). Three tanks were maintained at 28.5 ± 0.5 °C (control group, CO) while three tanks were passively cooled to 23 ± 0.5 °C (low temperature group, LT) over four hours. The LT group was maintained at 23 °C, as this was reported to be the lower thermal limit for embryonic development in zebrafish (Schirone and Gross, 1968) and therefore is the lowest temperature that could be applied from fertilization to adulthood (egg to egg) in a screen for temperature-sensitive mutations. However, more recent work has shown that zebrafish embryos can survive temperatures as low as 22 °C (Scott and Johnston, 2012). The previously mentioned studies have clearly demonstrated that zebrafish embryos survive at 22 – 23 °C; therefore, we chose to begin the low temperature treatment at the onset of exogenous feeding. This allowed us to directly compare the effects of low temperatures on growth after yolk absorption and the physiological transition to exogenous feeding.

2.2. Rearing larvae and juveniles

Fish were raised based on the method described by Dabrowski and Miller (2018) and Delomas and Dabrowski (2018). During the larval and early juvenile stages, fish were maintained in static water containers at low salinity (3 ppt), moderate turbidity (3 – 10 NTU) maintained with *Nannochloropsis* algae paste (Nanno 3600, Reed Mariculture, Campbell, CA, USA), and 24 L:0D photoperiod. The CO group was fed marine rotifers (*Brachionus plicatilis*) until 10 dpf, when the water volume in the tank was increased to 6 L (20 fish/L) and the diet was switched to *Artemia* nauplii. The CO group was maintained under these conditions until 21 dpf. At 21 dpf, 10 – 20 fish from each tank (both CO and LT groups) were measured and the CO group was stocked in a freshwater recirculating system at a density of 2 fish/L and with a 13 L:11D photoperiod. Diet for the CO group was transitioned to dry feed (Otohime B1/B2) supplemented with *Artemia* nauplii. The CO group was maintained under these conditions for the remainder of the experiment.

As fish development and metamorphosis are dependent upon growth (McMenamin et al., 2016), we standardized changes in husbandry based upon size and not time (age). The LT group was maintained under the larval and early juvenile conditions (low salinity, turbidity, continuous light) until 35 dpf, when they had attained approximately the same size as the CO group at 21 dpf (Table 1). Additionally, the LT group was fed marine rotifers until 13 dpf, when they had attained the same mean length as the CO group had at 10 dpf (8.7 ± 0.1 mm, n = 10 fish measured from the CO group at 10 dpf and from the LT group at 13 dpf), and then fed *Artemia* nauplii until 35 dpf. At 35 dpf, 20 fish from each of the LT tanks were measured and the water temperature was raised to 28.5 ± 0.5 °C over 8 h. Fish were then stocked in a freshwater recirculating system at a density of 2 fish/L and with a 13 L:11D photoperiod. Diet was transitioned to dry feed (Otohime B1/B2) supplemented with *Artemia* nauplii. The LT group was maintained under these conditions for the remainder of the experiment.

2.3. Sex identification and fertility testing

Once fish were large enough to determine their sex by examining external morphology (Parichy et al., 2009) (62 dpf for the CO group and 75 dpf for the LT group), the numbers of males and females in each tank were counted. Five males and five females from each tank were measured. Fertility tests were then performed to determine if the low temperature during early gonadal development had any effect on reproductive performance later in life.

A sample of males and females (three and four fish of each sex from CO and LT tanks, respectively) from each tank was tested for fertility. Each male and female was spawned separately with a test fish. Test fish were unrelated to the experimental fish and had previously been proven to have high fertility (over 90% fertilization rate when spawned