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Does Shaking Alter the Time of Hatching in Incubated Medaka (*Oryzias latipes*) Embryos?

M. González-Doncel, S. A. Villalobos, M. S. Okihiro, D. E. Hinton

Department of Anatomy, Physiology, and Cell Biology, School of Veterinary Medicine, University of California-Davis, Davis, CA 95616, USA

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The Japanese medaka (*Oryzias latipes*) has been shown to be an excellent model in early life stage (ELS) tests using embryos. In these tests, time of hatching is important in determining the maximum length of exposure to chemicals. Often, the time of hatching of non-exposed and exposed embryos may be asynchronous depending on whether the incubation is conducted still or if a large volume of exposure medium is used (personal observations in our laboratory). Therefore, it becomes important to make the time of hatching as uniform as possible without disturbing the process of egg development and eventually causing harm to the embryos. Hatching time in medaka and other fish is extremely variable, but neither the cause nor the function of this variability is well understood (Teather et al. 2000).

A literature review has revealed a limited number of studies providing information on the effects of embryo culture conditions on development and hatching. Marty et al. (1990) showed that variables such as temperature and available oxygen -when using sealed containers- could affect medaka hatching speed. Yamamoto (1975) and Yamagami (1973) reported hatching times of about a week or even less (6 days) when embryos were incubated at 28°C and 30°C, respectively. Along with increased temperature, Yamagami (1970) and Yasumasu et al. (1989) found that hatching was a more synchronous event when embryos were shaken. Furthermore, Belanger et al. (1990) observed asynchronous hatching after ELS tests using different types of containers. However, none of these researchers quantified the difference between the treatments.

This report studies the effects of shaking on incubated medaka embryos on both time and synchronicity of hatching. This should provide information useful for future standardization of ELS tests with medaka.

MATERIALS AND METHODS

Culture conditions for medaka broodstock were as described elsewhere (Marty et al. 1990). Fertilized eggs were collected from medaka females. Only those embryos at late morula (stage 9, or 5 h post-fertilization) were selected for the

Correspondence to: M. González-Doncel, Laboratory for Ecotoxicology, Department of the Environment, INIA, Ctra. de la Coruña, Km. 7.5, E-28040 Madrid, Spain

experiments, pooled and placed by stratified random assortment (Dowdy and Wearden 1991) in twenty milliliter scintillation vials (Kimble Inc.) or in petri dishes (60 x 15 mm, Ref. No. 351007, or 35 x 10 mm, Ref. No. 351008, Becton Dickinson Labware) and sealed with Teflon tape. In all experiments, the embryos were incubated in embryo rearing medium (ERM) (Kirchen and West 1976) as described below.

In one experiment, 80 individuals were split into two groups of vials, each consisting of 5 replicates (n= 8) with 2 ml of ERM. One group was shaken at 75 rpm (Junior orbital shaker, Lab Line Instruments, Inc.) until hatching occurred, and the other group was held still. Additionally, 100 individuals distributed in two sets of 5 vials each loaded with 10 embryos and 2.5 ml of ERM were used to test if shaking would have a similar effect in a more crowded container.

In the second set of experiments, 150 embryos were divided into four groups of petri dishes, each consisting of five replicates. Two groups included 10 embryos with 10 ml of ERM in large (60 x 15 mm) dishes. The other two groups consisted of 5 embryos with 2 ml of ERM in small (35 x 10 mm) dishes. One group of each type of petri dish was shaken at 75 rpm until hatching while the remaining corresponding groups were held still. Additionally, 150 embryos were grouped and incubated identically to the above 100 embryos although for this time scintillation vials were used.

In all experiments, the ERM solutions were replaced on day 7 for the shaken groups and on day 8 for the still groups. All embryos were kept at 25 ± 1°C with a 16 h light, 8 h dark (16L: 8D) photoperiod cycle. Identification of each stage attended to observations visible with a dissecting stereomicroscope at 70-80X. Staging, timing, and the terminology used herein followed primarily Iwamatsu (1994). Evaluations were made on a daily basis. The number of embryos was recorded as they hatched. Statistical differences at $P < 0.05$ between treatments were determined by the Student's t test and by analysis of variance (ANOVA) using a commercially available statistical software package.

RESULTS AND DISCUSSION

In the first experiments, the overall observed time of hatching for the shaken embryos was less than eight days (7.83 ± 0.11) while for the still embryos was slightly more than nine days (9.33 ± 0.29). Results for each replicate are presented in Table 1. Similar differences were observed when incubation conditions were modified to 10 embryos and 2.5 ml per vial. Hatching times were 7.80 ± 0.07 days and 9.03 ± 0.36 days for the shaken and still embryos, respectively (Table 2). For both incubation conditions there was less dispersion around the mean hatching time for the shaken embryos and differences proved statistically significant ($P < 0.05$, Student's t test) (Figure 1).

For the second set of experiments, Table 3 presents hatching time for eggs incubated in 2 or 10 ml using petri dishes or vials.

Table 1. Number of hatchlings per observed day in the shaken (Sh; 75 rpm) and still (St) vials containing 2 ml of incubation medium and 8 embryos per vial.

Day No.	Sh 1	Sh 2	Sh 3	Sh 4	Sh 5	St 1[a]	St 2	St 3	St 4	St 5
7	2	-	2	1	2	-	-	-	-	-
8	6	8	6	7	6	-	-	1	-	1
9	-	-	-	-	-	6	5	6	7	4
10	-	-	-	-	-	1	2	1	1	3
11	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	1	-	-	-

[a] One embryo accidentally lost, thus n=7.

Table 2. Number of hatchlings per observed day in the shaken (Sh; 75 rpm) and still (St) vials containing 2.5 ml of incubation medium and 10 embryos per vial.

Day No.	Sh 1	Sh 2	Sh 3	Sh 4	Sh 5	St 1[a]	St 2	St 3	St 4	St 5[b]
7	2	3	1	2	2	-	-	2	-	-
8	8	7	9	8	8	1	2	2	2	1
9	-	-	-	-	-	4	4	6	5	5
10	-	-	-	-	-	3	4	-	3	3
11	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-

[a] One dead embryo and one dead larva during development; larva presented anisophthalmia and lordosis. Thus n=8.

[b] One dead embryo during development presented congenital abnormalities. Thus n=9.

In all tests the mean hatching time for the shaken embryos was < 8 days with the exception of those incubated in vials with 10 ml. Significant differences were observed between each incubation treatment for the 2 ml experimental group (Table 3; $P < 0.05$, Student's t test). For the 10 ml group, average-hatching times differed significantly between petri dishes and the corresponding incubated vials (Table 3). Shaking in 2 ml, either in vials or in petri dishes, helped in synchronizing the hatching process (Figure 2).

Results from our studies agree with previous ELS assays such as those by Wisk and Cooper (1990) showing a significant overall difference in the number of days that medaka embryos took to hatch (6 ± 1.5 days) after incubated statically in vials with 1 ml at 25°C under a 16L: 8D regime. Using similar conditions, Teather et al. (2001) determined hatching times between 10.4 ± 0.97 and 10.7 ± 1.34 days.

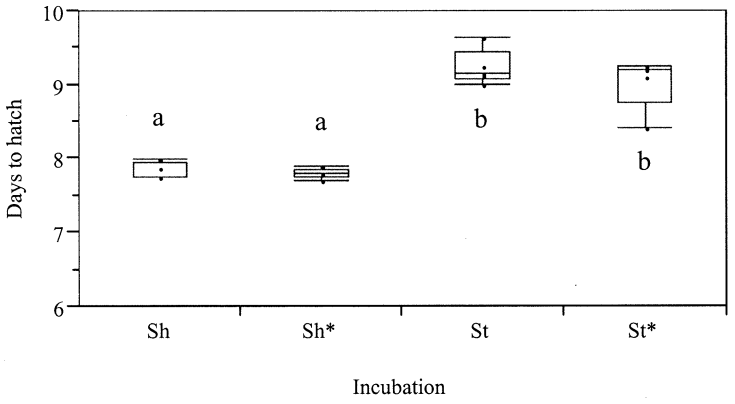

Figure 1. Median number of days (±75th and 95th percentiles) to hatch for embryos incubated in shaken (Sh; 75 rpm) or in still vials (St). Incubating conditions consisted of 2 ml and 8 embryos (Sh and St) or of 2.5 ml and 10 embryos (Sh* and St*) per vial. Values represent the response from 5 replicates. Different letters indicate significant differences ($P < 0.05$, Student's *t* test).

Table 3. Effects of agitation (shaken -Sh, 75 rpm-; or still -St-) on hatching time in medaka eggs incubated in petri dishes (PD) or vials (V)*.

Incubation	Time to hatch (days) (mean ± s.d.)
2 ml/5 embryos	
PD. Sh	7.12 ± 0.18^{a}
PD. St	8.40 ± 0.40^{b}
V. Sh	7.96 ± 0.22^{c}
V. St	8.93 ± 0.35^{d}
10 ml/10 embryos	
PD. Sh	7.50 ± 0.32^{a}
PD. St	8.30 ± 0.53^{b}
V. Sh	$8.81 \pm 0.50^{b,c}$
V. St	9.16 ± 0.34^{c}

* Values represent the mean (± s.d.) response from 5 replicates. Different superscript letters indicate statistical differences for a specific incubation among an experimental group ($P < 0.05$, Student's *t* test).

Earlier studies by Cloud (1981) reported hatching rates of 19.6 ± 1.1 days after static incubation with 10 ml of rearing medium in 25 ml Erlenmeyer flasks and at 22 ± 1°C and 16L: 8D.

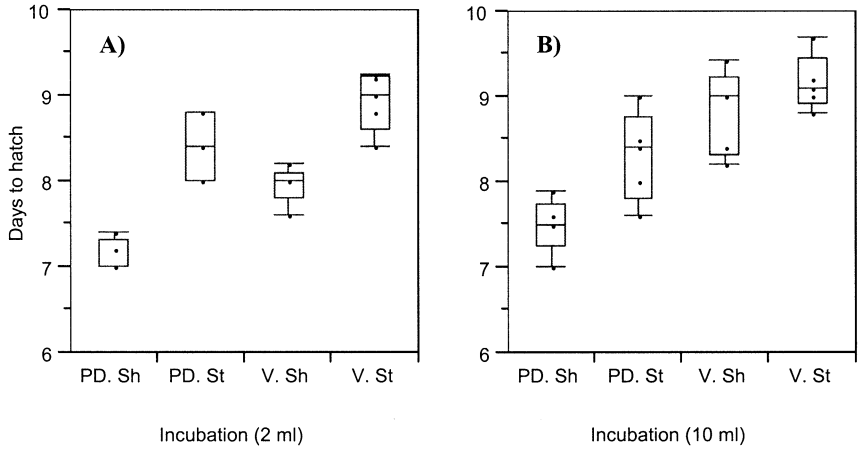

Figure 2. Median (±75th and 95th percentiles) number of days to hatch for embryos incubated in shaken vials (V. Sh) or petri dishes (PD. Sh) at 75 rpm, and in still vials (V. St) or petri dishes (PD. St). Incubating conditions consisted of: A) 2 ml ERM with 5 embryos per container, and; B) 10 ml ERM with 10 embryos per container. Values represent the response from 5 replicates.

In our studies shaking did not seem to alter the rate of initial development during the first four days since no morphological differences between either group were noticed until that day. However, after day 5 (≥ 120 h old embryos) it appeared that the rate of development increased in the groups of shaken embryos: earlier pigmentation (i.e., melanophores) and frayed yolk sac (quantitative data not included), and a larger swim bladder (Figure 3). In addition, the degradation of the inner layer of the chorion by the choriolytic enzymes -the last step before emergence (Yamagami 1997)- was observed earlier in the eggs shaken in which the chorion looked refractory and its transparency diminished. Likewise, in the shaken embryos at 168-192 h in development, the entire mouth (upper and lower jaw) was formed and the lower jaw moved. Under still conditions these features are characteristic in ≥ 192 h old embryos (Iwamatsu 1994). Interestingly, the exception concerned the shaken eggs incubated in vials with 10 ml where development was similar to those eggs incubated under still conditions.

From these results we can conclude that shaking the embryos not only shortens the time of hatching, but also makes this process and the embryonic development a more uniform event. Continuous movement of the medium may help in maintaining higher levels of dissolved oxygen, which in turn will increase physiological processes while speeding emergence. Results reported by Monroy et al. (1961) showed that the pattern of yolk utilization during medaka development followed quite closely the curve of oxygen uptake.

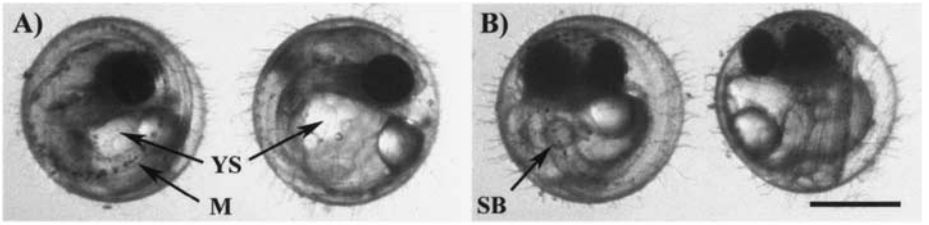

Figure 3. Advanced stage medaka embryos (approx. 120 h development at 25°C) incubated in vials with 2 ml ERM from hour 5 in development (stage 9). Right-located embryos in 3A (lateral-right view) and in 3B (dorsoventral view) were incubated still, while those at left were incubated shaken (75 rpm). Shaken embryos in 3A and 3B show the tip of the tail reaching the hind-brain region, a frayed yolk sac (YS), and the presence of strong pigmentation caused by melanophores (M) over the dorsal line. An enlarged swim bladder (SB) is also noticeable in the shaken embryo in 3B. Compare to still embryos in 3A and 3B. Bar = 0.5 mm.

Hishida and Nakano (1954) previously demonstrated that oxygen uptake in medaka embryos increases exponentially during the course of development. In our experiments, due to the high water column (> 20 mm) in the incubations using vials with 10 ml, the embryos, sitting on the bottom, may have lower dissolved oxygen levels available than those in shaken vials containing 2 ml where the water column is reduced to a mere 10 mm and where the ratio of water surface area to volume of medium is higher. Using adequate microprobes, ongoing studies will be conducted to check for dissolved oxygen levels in the shaken and in the still incubation containers. Agitation of the embryos could also contribute in homogenizing the choriolytic enzyme as there may be a better distribution thorough the inner layer of the chorion. Initially, this hypothesis could be discarded for the shaken embryos in vials and 10 ml. However, we observed that these individuals did not undergo as much movement over the bottom surface of the container as those incubated in containers holding a lower water column.

Under static laboratory conditions hatching of medaka eggs has been asynchronous (Belanger et al. 1990; Bentivegna and Piatkowski 1998). However, we have demonstrated that by shaking vials with 2 ml of incubation medium the hatching process is synchronized allowing establishing maximum exposure times as well as targeting into more specific developmental windows -stages- of exposure. Also, by shaking the containers, natural conditions may be better reflected since some degree of agitation is common in inhabiting natural waters of most fishes, including medaka. Conservative temperatures and shaking should reduce this time even more while synchronizing the embryo development. Further experiments should be conducted to examine these possibilities.

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