The effect of environmental stressors on the development and behaviour of larval *Oryzias latipes*

Emily McIntosh

Supervisor: Dr. Caleb T. Hasler

Committee members: Dr. Scott Forbes and Dr. Anuraag Shrivastav

A thesis submitted in partial fulfillment of the Honours Thesis (BIOL-4111/6) Course

Department of Biology

University of Winnipeg

2020

Abstract

Elevated water temperature and dissolved carbon dioxide (CO_2) are two environmental stressors that freshwater organisms face in the Anthropocene. Larval fishes are particularly susceptible to elevation in water parameters, as they are often confined to rearing habitats where temperature and CO_2 are nearing species-specific maxima. In this study, 240 freshwater Japanese medaka *(Oryzias latipes)* eggs were exposed to either control conditions (27˚C, ~ 500 µatm pCO_2), elevated water temperature (36˚C), elevated CO_2 (~1500 µatm pCO_2) or both elevated temperature and CO_2 (36˚C, ~1500 µatm pCO_2). Exposures were applied either during the early, middle or late developmental stages and the morphological and behavioural data was collected ten days post-hatch. I predicted that elevated temperature and CO_2 would decrease hatching success, and produce abnormalities in the swim bladder, spine or heart. In addition, I predicted that fish exposed to the stressors would show a change in swimming behaviour. Of the behavioural parameters observed, a significant difference was found in the distance travelled among the larval fish exposed to the treatments. There was no significant change between treatments or time intervals for hatching success, length or morphology. As rising CO_2 and warming are likely to have a consequential impact on freshwater species, further research dedicated to understanding the ramification of climate-induced stressors is imperative.

Acknowledgements

Firstly, I would like to thank my supervisor, Dr. Caleb Hasler, for his constant support and guidance throughout my research. I am grateful for everything I learned in the Hasler Lab and will treasure it for the rest of my scientific career. Thank you to my committee members, Dr.Scott Forbes and Dr. Anuraag Shrivastav, for their feedback and interest in my project. I would also like to thank my thesis coordinator, Dr. Jens Franck, for his time spent supporting my project.

Thank you to the animal care technicians, Robyn Cole and Dan Wasyliw for teaching me the medaka care protocols and the support throughout my research. I would also like to thank the Masters students in the Hasler lab; Jenna Fleet, Theresa Mackay and Jamie Card, for acting as my mentors.

Finally, I would like to thank my family and friends for enduring the countless pictures I have shown them of the medaka, and for their never-ending support during my studies.

Table of Contents

List of Figures

List of Tables

List of Appendices

1. Introduction

Anthropogenic CO_2 emitted by human activity is one of the greatest contributors to ocean acidification and warming (McNeil and Matsumoto 2019). It is estimated that air temperature and atmospheric CO_2 will increase between 2.6-4.8˚C and 420-940 ppm by 2100 (Collins *et al*., 2013; Porter *et al*., 2014). A symptom of rising CO_2 can be weak acidification in freshwater from CO_2 uptake that results in a small change in environmental pH by one or two units (Hasler *et al.,* 2018); however, biochemical and geological processes local to water bodies will also contribute to the level of future acidification (McNeil and Matsumoto 2019). To date, research concerned with climate change and aquatic species has been focused more on marine than freshwater systems (Ou *et al*., 2015; Hasler *et al*., 2016a; Jesus *et al*., 2018). Furthermore, few studies have aimed to understand how both warming and elevated CO_2 will influence freshwater biota (Hasler *et al.,* 2016b; 2018). Understanding how temperature and CO_2 paired as stressors influence freshwater biota is imperative to predict future responses of biological communities to environmental change.

Environmental change inherently affects freshwater fish populations (Hasler *et al.,* 2016a; Olusanya and van Zyll de Jong 2018). Fish are poikilothermic, and can be vulnerable to temperature-induced stress, as they are unable to regulate body temperature (Olusanya and van Zyll de Jong 2018; Trumbo *et al*., 2014). Elevated temperatures likely affect fish physiology through changes to metabolic demands (Nebauer and Andersen 2019). Changes in pH due to increasing levels of CO_2 have been shown to have adverse reactions on normal physiological functions in aquatic species (Wabnitz *et al.,* 2018). However, the severity of the responses will likely depend on the length of exposure and level of CO_2 (Kates *et al.,* 2012). Previous studies have suggested that a pH drop by one unit (7.0 to 6.4-6.0) induces mortality in freshwater fish

(Bernier and Randall, 1998; Fivelstad *et al.*, 2003). Other studies have observed a decline in hatching success and an increase in abnormal embryos (Forsgren *et al.,* 2013), as well as decreased growth from reduced foraging after high CO_2 exposure (Ishimatsu *et al.,* 2004). Environmental change affecting the physiology of fish is also reflected in their behaviour, as fish have restricted movement and there are limits to what they can behaviourally avoid (Hasler *et al*., 2018; Heuer *et al*., 2019). Therefore, fish are reliant on a consistent environment for normal growth, behaviour, and metabolism (Jesus *et al.,* 2018)

The freshwater Japanese medaka *(Oryzias latipes)* serves as a model organism to test how environmental change might affect freshwater fish because it has a short generation time, spawns frequently and has embryonic stages comparable to multiple species (Iwamatsu 2004; Kim *et al*., 2016). Of the 39 stages of medaka development outlined by Iwamatsu (2004), crucial stages to be noted are spine, heart and swim bladder development. Previous studies have argued that when exposed to poor rearing conditions, fish embryos may show abnormalities in these stages, have a reduced hatching success (Forsgren *et al*., 2013; Murray *et al*., 2019), and demonstrate altered swimming behaviour (Ishimatsu *et al*., 2008; Rossi *et al.,* 2015). High temperature may induce spinal defects (Ytteborg *et al*., 2010) or reduce swim bladder inflation (Trotter *et al*., 2003). Elevated levels of both temperature and CO_2 may reduce stroke volume in the heart (Ishimatsu *et al.,* 2004; For review see Keen *et al*., 2017). High CO_2 may decrease the heart rate (Reid *et al.,* 2000) or reduce swimming speeds (Rossi *et al.,* 2015). Research on the earliest stages of fish is important for understanding the full range of the effects of climate induced changes (Franke and Clemmesen 2011).

The objective of my research is to test if exposure to elevated temperature and elevated CO_2 during various embryonic stages has consequences for larval medaka morphology, growth and

behaviour. My study will aim to identify time-sensitive stages at which development will be hindered if eggs are exposed to both environmental stressors. I hypothesize that the hatching success of the eggs will be negatively affected and the larval fish will show abnormalities in the heart, spine and swim bladder. Additionally, I expect that swimming behaviour will be altered in the fish exposed to the treatments. With the data I have collected, I hope to emphasize the need for further research dedicated to climate-induced stressors and remediation.

2. Methods

2.1 Animal Husbandry

Adult medaka were kept in tanks of aquatic water at 27 ˚C and on an alternating 14-hour light and 10-hour dark cycle in the University of Winnipeg. Tanks were held in the Aquaneering© Zebrafish housing system, with constant circulating water throughout each of the racks. The adults were fed according to the University of Winnipeg standard operating protocol, with Zeigler Adult Zebrafish food containing Spirulina, Cyclop-Eeze© and Golden Pearl's larval diet. Hatched larval fish were held in larval tubes placed in the same housing system as the adults. Larval fish were fed four times a day with Paramecium and larval diet.

2.2 Egg rearing

Fertilized eggs were removed from fish tanks of approximately 5 males and 5 females, and placed in Petri dishes containing E2 embryonic development medium (see Appendix). Fifty percent of the E2 medium was replaced with fresh solution daily. The eggs were incubated at 29.5˚C and were observed for 6 days or until hatching. The Petri dishes were monitored daily to remove any hatched larvae or dead eggs, which were indicated by a white and opaque appearance (Iwamatsu 2004) or lacking a heartbeat in the case of the larvae.

2.3 Treatments

Sixty eggs per environmental treatment were acutely exposed to: normal water conditions, high temperature, high temperature and high CO_2, or high CO_2. Of the 60 eggs, the environmental stressors were either applied during the early, middle, or late developmental stages, with 20 eggs per developmental stage. Environmental stressors were applied by treating the E2 media, which then replaced the original media in the Petri dishes. To heat the E2 media to 36˚C, a beaker of the media was heated using a water bath and a hot plate. To elevate CO_2 in the E2 media, CO_2 was bubbled into a beaker of the media, lowering the pH to 6.8 (~1500 µatm). Control environmental values were 27˚C with a baseline pH between 7.0 and 7.5 (~500 µatm) depending on water conditions in the rack. The treatments were acute and applied daily until hatching.

2.4 Larval morphology, growth and behaviour

At ten days post-hatch, larval medaka were euthanized using an overdose of solution of MS222. Morphological features including swim bladder inflation, spinal deformities, and cardiac defects were monitored using a dissecting scope. Cardiac defects were defined as an abnormal heartbeat or a visible cardiac edema. Larval behaviour was observed by recording the fish swimming in a 5 cm container for three minutes. The length of the fish was measured using a still frame of the video recording. Distance swam, velocity, and time spent in the outer zone of the container was calculated using an automatic tracking software (Noldus Ethovision XT 14).

2.5 Statistical analyses

Two-way ANOVAs and a post hoc Tukey test were used to determine treatment level effects and were completed using R (R Core Team 2020). Visual inspection of residuals were used to validate models and significance was tested at $\alpha = 0.05$.

3. Results

3.1 Hatching success

Of the 240 fertilized eggs collected, 201 eggs hatched (Table 1). The overall survival rate was 83.75% among the four treatments (Table 2). In all of the treatments, the eggs hatched by Day 6, with the majority of eggs hatching on Day 5. No trend was established between the treatments.

Table 1. The number of dead eggs and hatched eggs removed from the incubating Petri dishes. The eggs hatched over a period of eight days.

		Dead eggs			**Hatched eggs**		
Treatment	**Day**	Early	Middle	Late	Early	Middle	Late
Control	1	0	0	0	0	0	0
	2	1	0	0	0	0	0
	3	0	0	4	0	0	0
	4	1	0	2	3	0	0
	5	0	0	1	10	20	4
	6	0	0	2	2	0	7
	7	0	0	0	0	0	0
	8	1	0	0	0	0	0
High CO2	1	0	0	4	0	0	0
	2	0	0	0	0	0	0
	3	1	0	1	0	0	0
	4	0	0	0	0	0	0
	5	0	0	0	7	17	7
	6	1	1	0	11	2	7
Temp x CO2	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	1	0	2	0	0	0
	4	1	3	0	1	5	0
	5	0	0	1	17	12	2
	6	0	0	0	0	0	15
High Temp	1	0	0	0	0	0	0
	2	4	0	0	0	0	0
	3	0	0	0	0	0	0
	4	2	0	0	3	0	0
	5	0	0	0	7	7	13
	6	0	1	1	4	12	6

	7	0	0	0	0	0
	8	1	0	0	0	0

Table 2. Survival rates of the eggs exposed to the four treatments at time intervals early, middle and late.

	Treatments			
Time	Control	High CO_2	High Temp	Temp x CO_2
Early	0.75	0.90	0.70	0.90
Middle	1.00	0.95	0.95	0.85
Late	0.55	0.70	0.95	0.85

3.2 Larval Growth

Length of the larval fish did not significantly differ among treatments or among time stages (Table 3; Figure 1). Non-significantly, larval growth was greatest in the high temperature group (6.46 mm ± 1.3; control: 6.35 mm ± 1.1; Figure 1), with the biggest larval fish observed in the middle time stage (6.68 mm± 0.5; control: 5.56 mm ± 0.9; Figure 1). The smallest fish were found in the high temperature and CO_2 treatments, with the smallest length in the middle time stage (5.55 mm ± 0.9; Figure 1).

Table 3. Two-way ANOVA values for length of the larval fish measured ten days post-hatch.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr (>F)**
Treatment	3	0.251	0.08362	1.80	0.149
Time	2	0.193	0.09660	2.08	0.128
Residuals	176	8.174	0.04644		

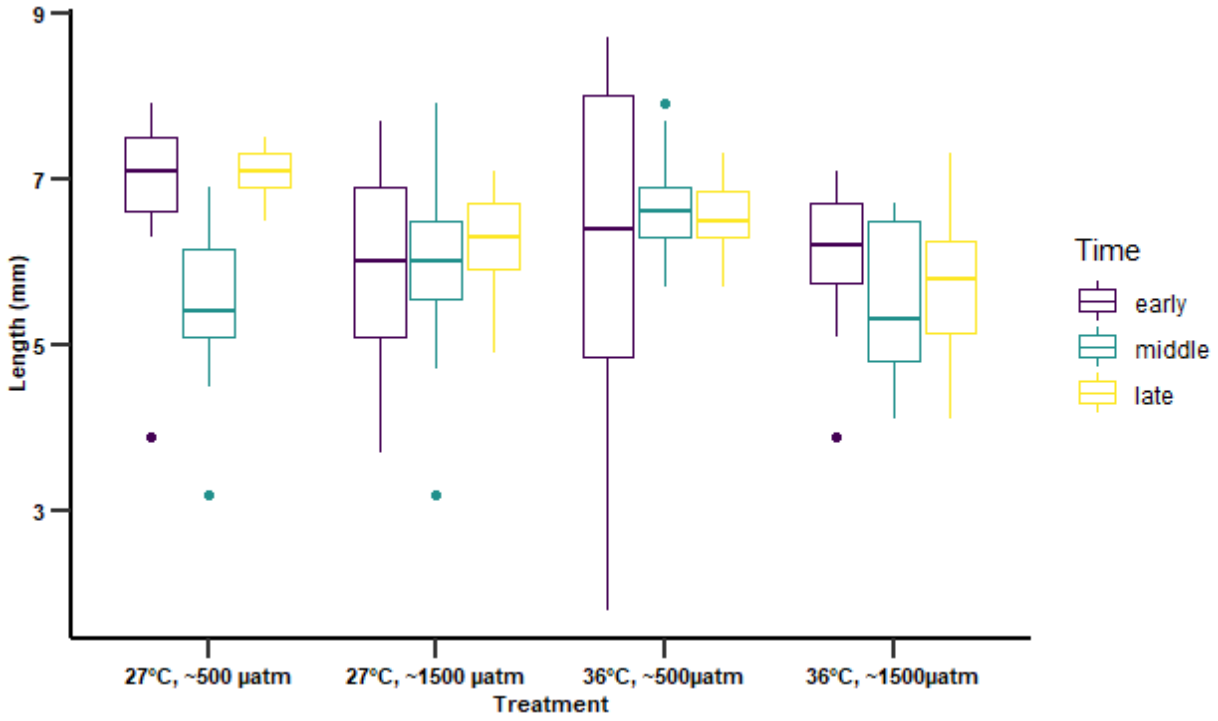

Figure 1. Length (mm) of the larval fish exposed to 27˚C and ~500 µatm, 27˚C and ~1500 µatm , 36˚C and ~1500 µatm or 36˚C and ~500 µatm during early, middle, or late developmental stages. Horizontal bars in the box plot represent the median response value, and the 75 and 25% quartiles. Whiskers represent ± 1.5 times the interquartile range, and outliers are indicated as dots.

3.3 Morphology

Of the 116 larval fish that survived rearing for ten days, 16 fish had visible abnormalities (Table 4). The most frequent abnormality was an absent swim bladder in the early stage fish. Few spinal defects (kinked and/or wavy) were observed across the larval fish, with the most in the early stage across all treatments. No cardiac defects were found in any of the other larval fishes. The high temperature group had the greatest number of abnormalities.

Table 4. Abnormalities observed in the hatched larval fish after exposure to the environmental stressors.

	Early			**Middle**			**Late**			
Treatment	ABS	SD	CD	ABS	SD	CD	ABS	SD	CD	**Total**
Control	1	0	0	1	1	0	2	0	0	**5**
High CO2	1	1	0	0	0	0	0	0	0	**2**
Temp x CO2	1	1	0	0	0	0	0	0	0	**2**
High Temp	2	2	0	1	0	0	0	2	0	**7**
Total	**5**	**4**	**0**	**2**	**1**	**0**	**2**	**2**	**0**	**16**

ASB: absent swim bladder; SD: spinal defect; CD: cardiac defect

3.4 Behaviour

A Two-Way ANOVA yielded a significant difference in the distance travelled between treatments, but not between time stages (Table 5). A post hoc Tukey test showed that the high temperature and high CO_2 group significantly differed from the high CO_2 group, and the high temperature group differed from the high CO_2 group (Figure 2). The mean distance travelled was greatest in the temperature treatments at 162 cm ± 108, with the greatest distance for the early time stage at 191 cm ± 175 (control: 122 cm ± 42; Figure 2). The larval fish in the CO_2 treatments travelled the shortest amount, at a mean of 120 cm ± 72, and a mean of 95 cm ± 28 for the middle time stage (control: 132 cm ± 61; Figure 2). Velocity of the larval fish and time spent in the outer area of the arena did not differ significantly among the treatments or time stages (Table 6). However, the following non-significant trends were observed. As demonstrated in Figure 3, the fastest moving fish were observed in the CO_2 treatments (1.19 cm/s ± 0.9;

control = 0.836 cm/s ± 0.3). Among the time stages, the fastest fish were in the late CO_2 group (1.60 cm/s ± 1.1; control = 1.05 cm/s ± 0.3). Slowest moving fish were identified in the early control time stage (0.683 cm/s ± 0.2). Figure 4 shows the time spent in the outer zone during the treatments. During the three minutes of recording, larval fish in all four treatments spent the most time in the outer zone of the arena (122 s ± 9.6). Fish in the middle temperature (147 s ± 15; control: 109 s ± 57) and late control groups (147 s ± 23) spent the most time in the outer zone. Fish in the early CO_2 time stage spent the least amount of time in the outer zone (93.7 s ± 46; control: 124 s ± 44).

Table 5. Two-way ANOVA values for distance travelled (cm) by the larval fish after exposure to the treatments.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr (>F)**
Treatment	3	2.828	0.9426	3.275	0.0239
Time	2	1.131	0.5656	1.965	0.1451
Residuals	108	31.082	0.2878		

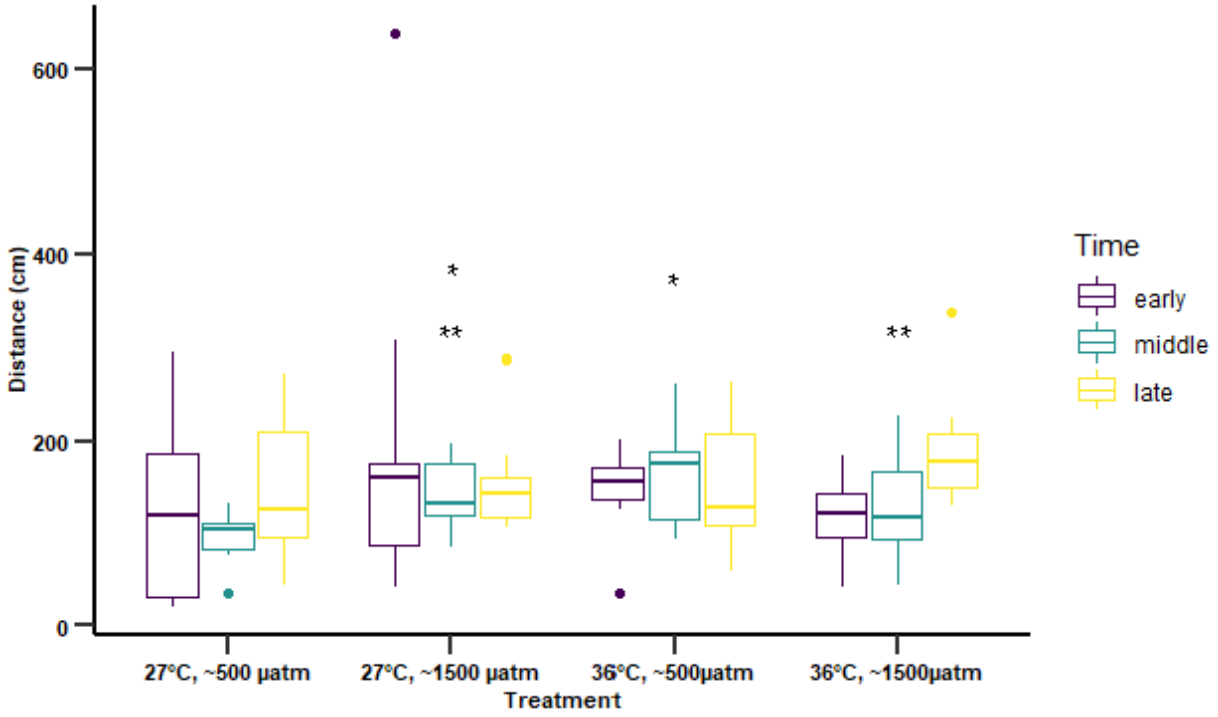

Figure 2. Distance travelled (cm) by the larval fish exposed to 27˚C and ~500 µatm, 27˚C and ~1500 µatm , 36˚C and ~1500 µatm or 36˚C and ~500 µatm during early, middle, or late developmental stages. Horizontal bars in the box plot represent the median response value, and the 75 and 25% quartiles. Whiskers represent ± 1.5 times the interquartile range, and outliers are indicated as dots. Asterisks denote significant differences between treatments (Tukey HSD post hoc test).

Table 6. Two-way ANOVA values for velocity (cm/s) of the larval fish after exposure to the treatments.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr (>F)**
Treatment	3	0.820	0.2733	1.067	0.362
Time	2	1.188	0.5940	2.340	0.101
Residuals	108	27.421	0.2539		

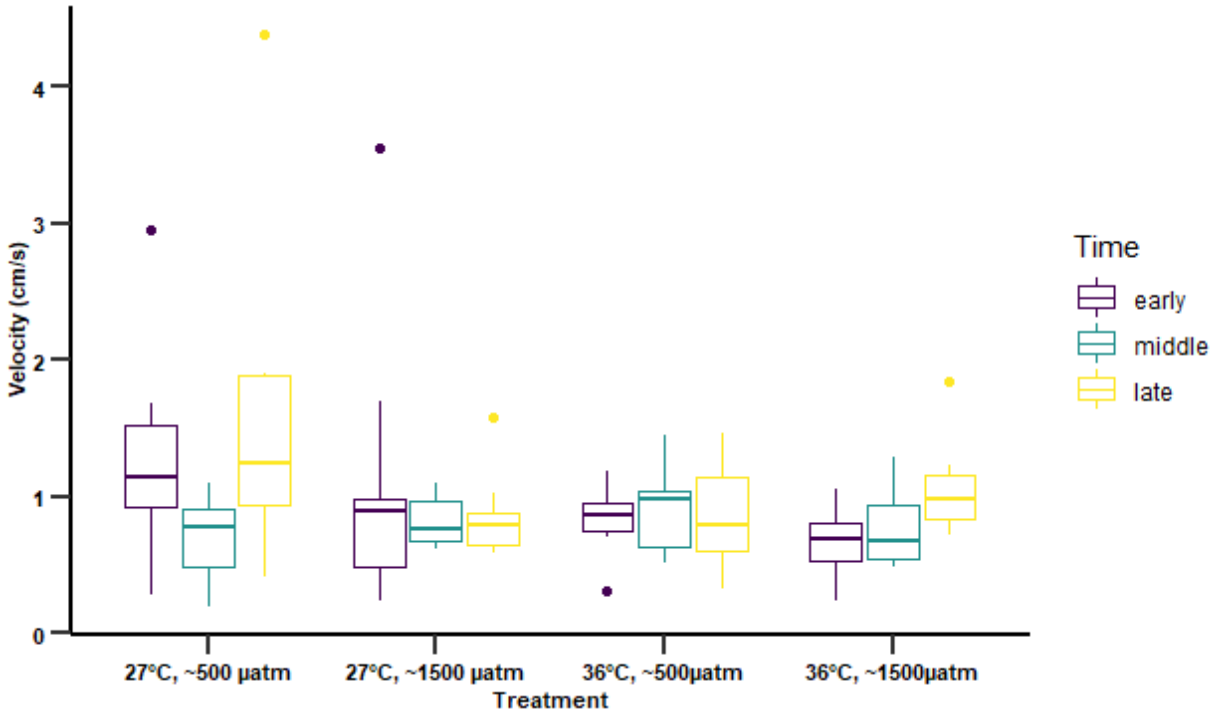

Figure 3. Velocity (cm/s) of the larval fish exposed to 27˚C and ~500 µatm, 27˚C and ~1500 µatm , 36˚C and ~1500 µatm or 36˚C and ~500 µatm during early, middle, or late developmental stages. Horizontal bars in the box plot represent the median response value, and the 75 and 25% quartiles. Whiskers represent ± 1.5 times the interquartile range, and outliers are indicated as dots.

Table 7. Two-way ANOVA values for time (s) spent in the outer zone of the arena after exposure to the treatments.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr (>F)**
Treatment	3	0.912	0.3041	1.937	0.128
Time	2	0.264	0.1320	0.841	0.434
Residuals	107	16.797	0.1570		

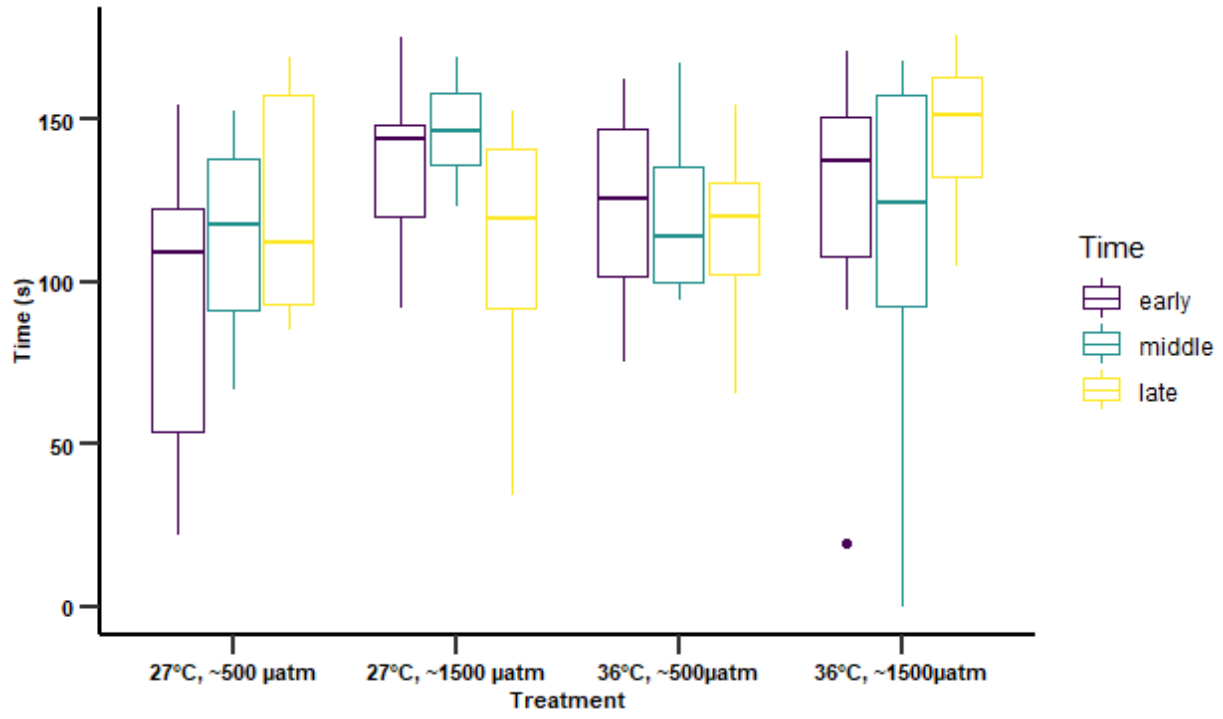

Figure 4. Time spent (s) in the outer zone of the arena of the larval fish exposed to 27˚C and ~500 µatm, 27˚C and ~1500 µatm , 36˚C and ~1500 µatm or 36˚C and ~500 µatm during early, middle, or late developmental stages. Horizontal bars in the box plot represent the median response value, and the 75 and 25% quartiles. Whiskers represent ± 1.5 times the interquartile range, and outliers are indicated as dots.

Discussion

4.1 Hatching success

From what has been observed in other studies (Forsgren *et al*., 2013; Murray *et al.*, 2019), I predicted that environmental stressors would decrease embryo survival. Neither elevated CO_2

nor temperature altered the hatching success of the embryos. My results agree with other high CO_2 studies (Franke and Clemmesen 2011; Wagner *et al*., 2002). When exposed to stressful conditions, medaka appear to adapt in such conditions due to their impressive temperature tolerance and ability for acid-base regulation in response to CO_2 exposure (Kirchmaier *et al*., 2015; Sampetrean *et al*., 2009; Sylvester 1972; Tseng *et al*., 2013). The wide temperature tolerance of medaka may have been the cause for the insignificant difference in hatching success for the elevated temperature treatments, as they can survive from 4-40˚C (Kirchmaier *et al*., 2015; Sampetrean *et al*., 2009). In addition, rather than the embryos being hindered by the rising temperature, some studies suggest that increased temperatures may induce a faster rate of development (Rosemore and Welsh 2012).

4.2 Larval Growth

Larval growth was not significantly influenced by elevated CO_2 or temperature ($p > 0.05$). This is in accordance with other studies on short-term high temperature exposures (Spinks *et al*., 2019) and long-term high CO_2 exposures (Cominassi *et al*., 2019; Sundin *et al*., 2019). Our findings disagree with studies on marine fish, who have found that both short-term and long-term exposures to high CO_2 induced a decrease in larval growth (Baumann *et al*., 2012; Fivelstad *et al*., 1998; Moran and Støttrup 2011). In addition, fish exposed to long-term high temperatures showed a decrease in growth (Spinks *et al*., 2019). Sundin *et al.* (2019) suggests that satiation influences fish growth, and fish not fed to satiation may show reduced lengths when exposed to environmental stressors. This may have affected the results in this study, as the larval fish were fed sufficient amounts of food. It can also be suggested that length was not affected by the environmental stressors because of the medakas acid-base regulation, high temperature tolerance

and ability to acclimate (Kirchmaier *et al*., 2015; Sampetrean *et al*., 2009; Sylvester 1972; Tseng *et al*., 2013).

4.3 Morphology

Spine development, cardiac development and the presence of a swim bladder were the chosen morphological parameters as they represented crucial stages of development (Iwamatsu 2004). I found no obvious relationship between high temperature and CO_2 exposures and abnormalities in the spine, heart or swim bladder. My results agree with those of Franke and Clemmesen (2011), who found no change in the occurrence of abnormal embryos in the Atlantic herring when exposed to high CO_2 (~4635 μatm). However, other studies across fish species have suggested that elevated temperatures reduced the number of vertebrae (Lindsey and Ali 1965), increased the presence of fused vertebrae (Ogawa 1965), reduced swim bladder inflation (Trotter *et al*., 2003), or reduced the stroke volume (Keen *et al*., 2017). Likewise, high CO_2 exposure decreased cardiac output and decreased the heart rate (Ishimatsu *et al.,* 2005; Reid *et al*., 2000). Based on our results, we can suggest that medaka may have the ability to acclimate to changes in the environment (Li *et al*., 2015), or change their morphology when exposed to environmental stress. Phenotypic plasticity in fish can be observed in other studies, such as remodeling the heart during early development of zebrafish to compensate for temperature-induced stress (Keen *et al*., 2017). Due to the wide range of results from exposure to environmental stressors, we can conclude that responses are highly variable among species, and species-specific research is necessary for definitive results (Franke and Clemmesen 2011).

4.4 Behaviour

I predicted that a change in pH from 7.8 to 6.8 may affect swimming behaviour in the medaka. Velocity and time spent in the outer zone did not differ significantly among treatments or time stages, however, larval fish in the high temperature and high temperature and CO_2 groups travelled significantly further than fish in the high CO_2 group. Research concerned with swimming activity after exposure to environmental stressors has produced a wide range of results across fish species. My results accord with other studies that have observed a reduction in swimming activity after exposure to elevated CO_2 (Regan *et al*., 2016; Schneider *et al*., 2019). In some studies, swimming activity increased (Biro *et al*., 2010; Fukuhara 1990), decreased (Johansen and Jones 2011), or did not change when exposed to high temperature (Kent and Ojanguren 2015). One hypothesis for the observed plasticity in temperature variation may be due to the variable body muscle mass after exposure to different temperatures (Sfakianakis *et al*., 2011). Elevated temperatures may result in thicker muscles for swimming, and therefore the ability to endure swimming in higher temperatures (Sfakianakis *et al.,* 2011). Where no change was observed, I can suggest that returning to baseline pH during the ten day rearing period mediated any disturbances to the physiology altering behaviour. This finding can be supported by previous studies suggesting that normal behaviour was observed once the fish were returned to baseline pH (Hasler *et al*., 2016b).

4.5 Limitations

Although research on environmental stressors and aquatic species is becoming more frequent, the type and length of exposure observed in my study on freshwater species in their embryonic stage was novel. The small sample size, short exposure time and the use of one generation proved to be limitations in this study. Statistical analyses with larger sample sizes would have

increased statistical power. The stressors were applied for brief periods at a time, rather than continuous exposure. After the E2 solution was treated and the original medium was replaced, we can assume that the heat eventually dissipated and the pCO_2 levels returned to baseline. In addition, only one generation was exposed to the treatments, so it is likely that greater results could be observed over a multi-generational scale. By using both the parent and offspring generations, nongenetic inheritance, or transgenerational plasticity may be observed in the ability to adapt to environmental stress (Bonduriansky and Day 2009; Schunter *et al*., 2016). Suggestions for future studies include long-term exposure throughout the egg phase and using multiple generations, as there is a lack of information concerning sustained exposure to CO_2 (Ishimatsu *et al*., 2004).

5. Conclusion

Elevated temperature and CO_2 did not affect hatching success, the occurrence of morphological abnormalities, larval growth or swimming behaviour in the Japanese medaka. Lethal exposure to both temperature and CO_2 varies among fish species (Ishimatsu *et al*., 2005), and it is likely the combination of multiple environmental stressors and longer exposures that will elicit a developmental and behavioural change in freshwater fish. I suggest that responses to environmental stressors are highly species-specific (Franke and Clemessen 2011; Murray *et al*., 2014), and gradual changes to the climate will directly affect freshwater fish. Future studies on long-time exposures and stressors applied during the developmental stages are imperative.

Literature Cited

Baumann, H., Talmage, S.C., and Gobler, C.J. 2012. Reduced early life growth and survival in a fish in direct response to increased carbon dioxide. Nat. Clim. Chang. **2**: 28-41

Bernier, N. J. and Randall, D.J.1998. Carbon dioxide anaesthesia in rainbow trout: effects of hypercapnic level and stress on induction and recovery from anaesthetic treatment. J. Fish. Biol. **52**:621–637.

Biro, P.A., Beckmann, C., and Stamps, J.A. 2010. Small within-day increases in temperature affects boldness and alters personality in coral reef fish. Proc. R. Soc. B. **277**: 71-77

Bonduriansky, R., and Day, T. 2009. Nongenetic Inheritance and Its Evolutionary Implications. Annu. Rev. Ecol. Evol. Syst. **40**: 103-25

Collins, M., R. Knutti, J. Arblaster, J.-L. Dufresne, T. Fichefet, P. Friedlingstein, X. Gao, W.J. Gutowski, T. Johns, G. Krinner, M. Shongwe, C. Tebaldi, A.J. Weaver, and M. Wehner. 2013. Long-term climate change: Projections, commitments and irreversibility. In *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. T.F. Stocker, D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Doschung, A. Nauels, Y. Xia, V. Bex, and P.M. Midgley, Eds. Cambridge University Press, pp. 1029-1136, doi:10.1017/CBO9781107415324.024

Cominassi, L., Moyano, M., Claireaux, G., Howald, S., Mark, F.C., Zambonino-Infante, J.-L.,Le Bayon, N., and Peck, M.A. 2019. Combined effects of ocean acidification and temperature on larval and juvenile growth, development and swimming performance of European sea bass (*Dicentrarchus labrax*). PLoS ONE. **14**: e0221283

Fivelstad, S., Haavik, H., Løvik, G., and Olsen, A. B. 1998. Sublethal effects and safe levels of carbon dioxide in seawater for Atlantic salmon postsmolts (*Salmo salar* L.): ion regulation and growth. Aquaculture **160**: 305-316

Fivelstad, S., R. Waagbø, S. F. Zeitz, A. Camilla, D. Hosfeld & A. Berit, 2003. A major water quality problem in smolt farms: combined effects of carbon dioxide, reduced pH and aluminium on Atlantic salmon (*Salmo salar* L.) smolts: physiology and growth. Aquaculture **215**: 339–357.

Forsgren, E., Dupont, S., Jutfelt, F. and Amundsen, T. 2013. Elevated CO2 affects embryonic development and larval phototaxis in a temperate marine fish. Ecol. Evol. **3**:3637-3646

Franke, A. and Clemmesen, C. 2011. Effect of ocean acidification on early life stages of Atlantic herring (*Clupea harengus* L.), Biogeosciences **8**: 3697–3707

Fukuhara, O. 1990. Effects of temperature on yolk utilization, initial growth, and behavior of unfed marine fish-larvae. Mar. Biol. **106**: 169-174

Hasler, C.T., Butman, D., Jeffrey, J.D., and Suski, C.D. 2016a. Freshwater biota and rising pCO_2? Ecol. Lett. **19**: 98-108

Hasler, C. T., Midway, S.R., Jeffrey, J.D., Tix, J.A., Sullivan, C., and Suski, C.D. 2016b. S. R. Exposure to elevated pCO2 alters post-treatment diel movement patterns of largemouth bass over short time scales. Freshwater Biol. **61**: 1590–1600.

Hasler, C.T, Jeffrey, J.D., Schneider, E.V.C., Hannan, K.D., Tix, J.A. and Suski, C.D. 2018. Biological consequences of weak acidification caused by elevated carbon dioxide in freshwater ecosystems. Hydrobiologia. **806**:1-12.

Heuer, R.M., Hamilton, T.J and Nilsson, G.E. 2019. The physiology of behavioral impacts of high CO2. Fish Physiol. 37: 1-33.

Huang, Q., Fang, C., Wu, X., Fan, J. and Dong, S. 2011. Perflurooctane sulfonate impairs the cardiac development of a marine medaka (*Oryziasmelastigma*). Aquat. Toxicol. **105**:71-77

Ishimatsu A, Kikkawa T, Hayashi M, Lee KS, and Kita J 2004. Effects of CO_2 on marine fish: larvae and adults. J. Oceanogr. **60**:731–74

Ishimatsu, A., Hayashi, M., and Lee, K-S. 2005. Physiological effects on fishes in a high CO_2 world. J. Geophys. Res. **110**: C09S09

Ishimatsu, A., Hayashi, M., Kikkawa, T. 2008. Fishes in high-CO_2, acidified oceans. Mar. Ecol. Prog. Ser. **373**: 295-302

Iwamatsu, T. 2004. Stages of normal development in the medaka *Oryzias latipes*. Mech. Dev. **121**:605-618

Jesus, T.F., Rosa, I.C., Repolho, T., Lopes, A.R., Pimentel, M.S., Almeida-Val, V.M.F., Coelho, M.M., and Rosa, R. 2018. Different ecophysiological responses of freshwater fish to warming and acidification. Comp. Bio. Phys, Part A. **216**:34-41

Johansen, J.L., and Jones, G.P. 2011. Increasing ocean temperature reduces the metabolic performance and swimming ability of coral reef damselfishes. Glob. Chang. Biol. **17**: 2971-2979

Kates, D., Dennis, C., Noatch, M.R., and Suski, C.D. 2012. Responses of native and invasive fishes to carbon dioxide: potential for a nonphysical barrier to fish dispersal. Canadian J. Fish. Aquat. Sci.**69**: 1748–1759

Kent, M., and Ojanguren, F. 2015. The effect of water temperature on routine swimming behaviour of new born guppies (*Poecilia reticulata*). Biol. Open **4**: 547-552

Keen, A.N., Klaiman, J.M., Shiels, H.A., and Gillis, T.E. 2017. Temperature-induced cardiac remodeling in fish. J. Exp. Biol. **220**: 147-160

Kim, B-M., Kim, J., Choi, I-Y., Raisuddin, S., Au, D.W.T., Leung, K.M.Y., Wu, R.S.S. and Lee, J-S. 2016. Omics of the marine medaka (*Oryzias melastigma)* and its relevance to marine environmental research. Mar. Environ. Res. **113**:141-152

Kirchmaier, S. Naruse, K., Wittbrodt, J., and Loosli, F. 2015. The genomic and genetic toolbox of the teleost medaka (*Oryzias latipes*). Genetics. **199**: 905-918

Li, A.J., Leung, P.T.Y., Bao, V.W.W., Lui, G.C.S., and Leung, K.M.Y. 2015. Temperature dependent physiological and biochemical responses of the marine *medaka Oryzias melastigma* with consideration of both low and high thermal extremes. J. Therm. Biol. **54**: 98-105

Lindsey, C.C., and Ali, M.Y. 1965. The effect of alternating temperature on vertebral count in the medaka (*Oryzias latipes*). Can. J. Zoo. **43**: 99-104

McNeil, B.I. and Matsumoto, K. 2019. The changing ocean and freshwater CO2 system. Fish Physiol. **37**: 1-32

Moran D., and Støttrup., J.D. 2011. The effect of carbon dioxide on growth of juvenile Atlantic cod *Gadus morhua* L. Aquat.Toxicol. **102**: 24-30.

Murray, C.S., Malveszzi, A., Gobler, C.J., and Baumann, H. 2014. Offspring sensitivity to ocean acidification changes seasonally in a coastal marine fish. Mar. Ecol. Prog. Ser. **504**: 1-11

Murray, C.S., Wiley, D., and Baumann H. 2019. High sensitivity of a keystone forage fish to elevated CO2 and temperature. Conserv Physiol. **7**: coz 084

Neubauer, P., and Andersen, K.H. 2019. Thermal performance of fish is explained by an interplay between physiology, behaviour and ecology. Conserv. Physiol. **7**: coz025; doi:10.1093/conphys/coz025.

Ogawa, N.M. 1965. A phenogenetic study of the vertebral fused (f) in the medaka, *Oryzias latipes*. Embryologica **9**: 13-33

Olusanya, H.O., and van Zyll de Jong, M. 2018. Assessing the vulnerability of freshwater fishes to climate change in Newfoundland and Labrador. PLoS ONE. **13**: e0208182

Ou, M., Hamilton, T.J., Eom, J., Lyall, E.M., Gallup, J., Jiang, A., Lee, J., Close, D.A., Yun, S S., and Brauner, C.J. 2015. Responses of pink salmon to CO_2-induced aquatic acidification. Nat. Clim. Chang. **5**:950-957

Porter, J.R., Xie, L., Challinor, A.J., Cochrane, K., Howden, S.M., Iqbal, M.M., Lobell, D.B. & Travasso, M.I. 2014. Food security and food production systems. In C.B. Field, V.R. Barros, D.J. Dokken, K.J. Mach, M.D. Mastrandrea, T.E. Bilir, M. Chatterjee, K.L. Ebi, Y.O. Estrada, R.C. Genova, B. Girma, E.S. Kissel, A.N. Levy, S. MacCracken, P.R. Mastrandrea & L.L. White, eds. Climate change 2014: impacts, adaptation, and vulnerability. Part A: global and sectoral aspects, pp. 485–533. Contribution of Working

Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge, UK, and New York, USA, Cambridge University Press.

R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Regan, M. D., A. J. Turko, J. Heras, M. K. Andersen, S. Lefevre, T. Wang, M. Bayley, C. J. Brauner, T. T. do Huong, N. T. Phuong & G. E. Nilsson, 2016. Ambient CO2, fish behaviour and altered GABAergic neurotransmission: exploring the mechanism of CO2 altered behaviour by taking a hypercapnia dweller down to low CO2 levels. J. Exp. Biol. **219**: 109–118.

Reid, S.G, Sundin, L., Kalinin, A.L., Rantin, F.T., and Milsom, W.K. 2000. Cardiovascular and respiration reflexes in the tropical fish, traira (*Hoplias malabaricus*): CO_2/pH chemoresponses. Respir. Physiol. **120**: 47-59

Rosemore, B.J., and Welsh, C.A. 2012. The effects of rearing density, salt concentration, and incubation temperature on Japanese medaka (Oryzias latipes) embryo development. Zebrafish **9**: 185-190.

Rossi, T., Nagelkerken, I., Simpson, S.D., Pistevos, J.C.A., Watson, S.-A., Merillet, L., Fraser, P., Munday, P.L., and Connell, S.D. 2015. Ocean acidification boosts larval fish development by reduces the window of opportunity for successful settlement. Proc. R. Soc. B. **282**: 20151954

Sampetrean, O., Iida S.., Makino S., Matsuzaki Y., Ohno K., and Saya, H. 2009.Whole organism cell cycle arrest in a living vertebrate. Cell Cycle **8**: 620–627.

Schneider, E.VC., Hasler, C.T., and Suski, C.D. 2019. Swimming performance of a freshwater fish during exposure to high carbon dioxide. Environ. Sci. Pollut. **26**: 3447-3454

Schunter. C., Welch, M.J., Ryu, T., Zhang, H., Berumen, M.L., Nilsson, G.E., Munday, P.L., and Ravasi, T. 2016. Molecular signatures of transgenerational response to ocean acidification in a species of reef fish. Nat. Clim. Chang. **6**: 1014 – 1018.

Sfakianakis, D.G., Leris, I., and Kentouri, M. 2011. Effect of developmental temperature on swimming performance of zebrafish (*Danio rerio*) juveniles. Environ. Biol. Fishes. **90**: 421-427

Spinks, R.K., Munday, P.L., and Donelson, J.M. 2019. Developmental effects of heatwave conditions on the early life stages of a coral reef fish. J. Exp. Biol. **222**: eb202713. doi:10.1242/jeb.202713

Sundin, J., Amcoff, M., Mateos-Gonzales, F., Raby, G.D., and Clark, T.D. 2019. Long-term acclimation to near-future ocean acidification has negligible effects on energetic attributes in a juvenile coral reef fish. Oecologia **190**:698 – 702

Sylvester, J.R. 1972. Possible effects of thermal effluents on fish: a review. Environ. Pollut. **3**: 205-215

Trotter, A.J., Pankhurst, P.M., Morehead, D.T., and Battaglene, S.C. 2003. Effects of temperature on initial swim bladder inflation and related development in cultured striped trumpeter (*Latrislineata).*Aquaculture. **221**:141-156

Trumbo B. A, Nislow K. H, Stallings J, Hudy M, Smith E. P, Kim D. Y, Wiggins, B., and Dollof, C.A. 2014. Ranking site vulnerability to increasing temperatures in southern Appalachian Brook Trout streams in Virginia: an exposure-sensitivity approach. Trans. Am. Fish. Soc. **143**:173–87

Tseng, Y-C., Hu, M.Y., Stumpp, M., Lin, L-Y., Melzner, F., Hwang, P.P. 2013. CO2-driven seawater acidification differentially affects development and molecular plasticity along life history of fish (Oryzias latipes). Comp. Biochem. Physiol. A. Mol. Integr. Physiol. **165**: 119-130

Wabnitz, C.C.C., Lam, V.W.Y., Reygondeau, G., Teh, L.C.L., Al-Abdulrazzak, D., Khalfallah, M.,Pauly, D., Deng Palomares, M.L., Zeller, D. and Cheung, W.W.L. 2018. Climate change impactson marine biodiversity, fisheries and societies in the Arabian Gulf. PLoS ONE.**13**: e0194537

Wagner, E., R. Arndt, and B. Hilton .2002. Physiological stress responses, egg survival and sperm motility for rainbow trout broodstock anesthetized with clove oil, tricaine methanesulfonate or carbon dioxide, Aquaculture. **211**: 353–366.

Ytteborg, E., Baeverfjord, G., Torgersen, J., Hjelde, K., and Takle, H. 2010. Molecular pathology of vertebral deformities in hyperthermic Atlantic salmon (*Salmo salar*). BMC Physiol. **10:** doi: 10.1186/1472-6793-10-12

Appendix I

Embryonic E2 Medium

Buffer Mix preparation

Dissolve reagents below in 1L of water and filter sterilize.

Reagent	Desired Concentration (mM)	Weight of salt (g)
KH_2PO_4	750	102.1
Na_2HPO_4	250	67.0

E2A preparation

Dissolve reagents in 2L of water and filter sterilize.

Reagent	Desired Concentration	Weight of salt (g)
NaCl	1.5 M	175
KCl	50 mM	7.5
$MgSO_4$ 7(H_2O)	100 mM	49.3
KH_2PO_4	15 mM	4.08
Na_2HPO_4	5 mM	1.42

E2B preparation

Dissolve reagents in 1L of water and filter sterilize.

Reagent	Desired concentration (mM)	Weight of salt (g)
$CaCl_2$ 5(H_2O)	500	73.5

E2C preparation

Dissolve reagents in1L of water and filter sterilize.

Reagent	Desired concentration (mM)	Weight of salt (g)
$NaHCO_3$	300	14.7

Final E2 medium

Add all solutions. Adjust pH to 7.2-7.6. Store at 28˚C. Expires one week after preparation.

Reagent	Volume (mL)
E2A	100
E2B	20
E2C	20
0.1% methylene blue	10

Appendix II

Significant developmental stages in *Oryzias latipes.* Adopted from Iwamatsu (2004).

Stage Interval	Key developmental features
Stage 22	First appearance of the tubular heart
Stage 24	Start of heart beating
Stage 26	Vacuolization of the notochord

Stage 32	Formation of the swim bladder
Stage 33	Completion of notochord vacuolization
Stage 36	Heart formation complete
Stage 39	Hatching stage

Appendix III

MS-222

Dissolve reagents in 500 mL of aquatic water. Store securely.

Reagent	Weight (g)
MS-222	1.0
$NaHCO_3$	0.5