Cochineal Dye Concentration and Treatment Time for Otolith Marking of Japanese Smelt Hypomesus nipponensis Embryos

Title: Cochineal dye concentration and treatment time for otolith marking of Japanese smelt embryos

Running head: Cochineal dye marking of Japanese smelt

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Abstract The main purpose of this study is to find the optimal cochineal dye concentration and treatment time for marking Japanese smelt *Hypomesus nipponensis*, more specifically their eggs, by understanding the mechanisms that determine survival rates and mark quality. Eggs were immersed in a range of cochineal dye concentrations (40-100 g/L) at varying intervals (6-72 h). Following that, mark quality was evaluated according to fluorescence intensity by examining the marked otolith of larvae. Statistical model selection indicates that the interaction between cochineal dye concentrations and immersion intervals (g/L × h) had a negative effect on survival, wherein at low concentrations, survival decreased with increasing immersion time but, at high concentrations, survival was relatively low at all immersion times. On the other hand, the results of evaluating marked otoliths indicate that mark quality improves with increases in concentration and immersion time individually. These results suggest there is a trade-off between mark quality and survival of Japanese smelt when using a cochineal dye solution. Combining the results of the survival rate and mark quality experiments, the cochineal dye marking conditions with both high survival rates and high fluorescence intensity are 60 g/L for 24 h, 40 g/L for 48 h and 40 g/L for 36 h.

Keywords Wakasagi · Immersion · ALC · Eyed eggs · Survival

41 Japanese smelt *Hypomesus nipponensis* inhabits fresh, brackish and coastal waters
42 throughout the Japanese archipelago (Hamada 1961; Saruwatari et al. 1997) and is one of the
43 most important commercial species for Japanese freshwater fisheries. Eyed eggs of Japanese
44 smelt have been transplanted into numerous lakes, ponds and reservoirs throughout Japan.
45 However, because the tagging techniques for Japanese smelt are still in early stages of
46 development, effectiveness of their propagation is still not clear. In North America, pond smelt
47 *Hypomesus olidus* as a closely related species of Japanese smelt eggs transported in the 1950s
48 established a breeding population in the San Juaquin estuary in California (Wales 1962). Dill and
49 Cordone (1997) note that it was also discovered in brackish waters, threatening the survival of
50 the imperiled delta smelt. These examples in Japan and North America exhibit the importance of
51 tracking pond smelt and evaluating their dynamics and recruitment for management purposes.
52 In order to conduct research on fish dynamics and recruitment, marking and recapture
53 programs would first need to be performed (Brown et al. 2002; Taylor et al. 2005; Baer and
54 Rösch 2008). Many methods of marking fish at different stages of their life cycle have been
55 developed. However, larvae are too small to be marked externally (e.g. fin clipping) or internally
56 (e.g. passive integrated transponder), and handling them individually would require large effort.
57 Genetic tags are not suitable for recapture processes due to high operating costs. Otolith thermal
58 marking could generally be considered as a viable option because of its common use in
59 hatcheries for salmon marking (e.g. Hagen et al. 1995; Volk et al. 1999), but not in the case of
60 Japanese smelt because its otolith is too small for marking codes to be reliably verified
61 (Hoshikawa unpublished data). Moreover, the method used for marking must be suitable for
62 application to large numbers of small fish, handling stress should be kept at a minimum, and the
63 marking and recovery procedures should be easy to perform (Skov et al. 2001). Thus far,
64 chemical marks, as internal–external marks have proven to be the most appropriate method for
65 evaluating large-scale stocking of small juveniles (Brown et al. 2002; Simon and Dörner 2005;
66 Baer and Rösch 2008).

Fluorochrome labeling dyes visibly mark otoliths, scales and other bony structures based on differential staining. Gelsleichter et al. (1997) investigated different methods for introducing markers to both teleosts and elasmobranchs. When choosing a marking method, one has to consider the stage of fish development, the environment (marine or freshwater) and the preferred mode of application (Lagardère et al. 2000), such as injection (Monaghan 1993), dietary intake (Honeyfield et al. 2006) and immersion. Some fluorochrome labeling dyes, such as alizarin red S (e.g. Eckman 2003; Crook et al. 2009) and alizarin complexone (ALC) (e.g. van der Walt and Faragher 2003), have been shown to be effective in producing fluorescent marks with few negative effects on survival. However, it was suggested that the Madder Color (MD) extracted from the roots of *Rubia tinctorum* L., which has alizarin as its main component, has negative effects on the health of rats (Inoue et al. 2009a, b). In fact, MD has been prohibited from use as an additive in food since 2004 in Japan. For these reasons, more investigation is required regarding the effects of alizarin on animal health. In the meantime, it might be necessary to develop marking techniques using alternative chemical dyes.

Cochineal dye is a natural red colorant extracted from dried female cochineal insects *Dactylopius coccus*, which are parasites of cacti native to Central and South America, particularly Peru. Cochineal dye is used worldwide as a coloring agent in foods, drinks, cosmetics, quasidrugs, and drugs (Ohgiya et al. 2009). Recently, cochineal dye has been attracting attention as a labeling agent for marking fish, especially for small fish like the Japanese smelt that can be consumed by humans in one go. The fish are marked by immersing their fertilized eggs or larvae in a labeling liquid, wherein the labeling agent is dissolved, staining the hard tissue (otolith, spine, scale, etc.). Cochineal dye is suitable for labeling red sea bream *Pagrus major*, sea urchin (class *Echinoidea*), bastard halibut (also known as Olive Flounder) *Paralichthys olivaceus* and Japanese pufferfish *Takifugu rubripes* (Kengo Ohta, Japan Patent Kokai 2008-67648). However, thus far, there are no studies evaluating mark quality and mortality induced by cochineal dye on Japanese smelt as a freshwater fish.

ALC studies have shown that concentration and immersion intervals are determining factors in regard to mark quality and survival (Iglesias and Rodriguez-Ojea 1997). If the same is true of cochineal dye, then it will be necessary to first understand how these factors contribute in a cochineal dye marking process. The possible outcomes in regard to concentration and immersion time contributing to mark quality and survival are as follows: 1. neither factor contributes; 2. only one of them contributes; 3. both of them contribute individually; 4. they create an interaction. Without first knowing which one of these is the case for cochineal dye, it would be very difficult to find the optimal marking condition for it. Therefore, the main aim of the current study was to find the optimal concentration and treatment time for cochineal dye marking of Japanese smelt by understanding the mechanisms which determine survival rates and mark quality.

METHODS

Test eggs

Artificial fertilization of eggs was carried out by the Lake Suwa Fisheries Cooperative on April 14 (male to female ratio = 23:1; total egg weight = 2, 780 g) and 15 (male to female ratio = 22:3; total egg weight = 4, 320 g), 2018 using mature fish caught from the Togawa River. Egg adhesive removal (Izuka 2005) was performed by putting the eggs in a 0.05 % tannic acid solution and stirring it for 10 minutes. Following that, the eggs were put into tanks with a constant flow of groundwater, at about 12℃, through a VU300 vinyl chloride pipe installed in the facility of the fishery cooperative. The eggs were refrigerated and transported to the Suwa branch of the Nagano Prefectural Fisheries Experimental Station on April 23, 2018 and were tested on the same day.

Labeling liquid

Carmine Red MK-40 manufactured by Kiriya Chemical Co., Ltd. was used as the cochineal dye.

One of the control groups was marked using ALC manufactured by Dojindo Laboratories Co., Ltd. The second, and last, of the control groups was marked simply by using ordinary dechlorinated tap water. The main reason why we had an ALC control group was because ALC is commonly used for the marking of fish. The water control group, on the other hand, mainly served as an equipment check (whether or not the microscope was correctly reading fluorescence levels) and to set the standard for invisible mark quality. The cochineal dye was dissolved in boiling dechlorinated water at 80℃ or higher to a predetermined concentration and stirred for 1 hour on a magnetic stirrer hot plate (REXIM RSH-1AN manufactured by AS ONE Corporation). After cooling, the solutions were readjusted by adding water to replace the amounts lost through the process of evaporation. The ALC solution was prepared using a conventional method to a concentration of 0.1 g/L (Tomoda and Kuwata 2006). The solutions were stored in 8℃ incubators until testing and stirred again just before use.

Marking

Live eyed eggs were placed in petri dishes (φ90 × 15mm) filled with different solutions for varying intervals of time. The petri dishes were then placed in incubators set at 8℃ without any light sources until the end of the entire marking procedure. Each petri dish had 50 eggs and 25 ml of the following solutions: cochineal dye (40, 50, 60, 70, 80, 90, 100 g/L), ALC (0.1 g/L), and dechlorinated water (0 g/L). Aeration of the eggs was deemed unnecessary for this study due to an adequate supply of oxygen in the petri dishes, as was confirmed by a preliminary test. The immersion intervals for the cochineal dye solutions were 6, 12, 24, 36, 48, 60 and 72 hours, while for the control ALC and water solutions, they were simply 24 and 72 hours respectively. Based on the results of a preliminary test, which was conducted to determine what combinations of concentration and immersion time are lethal for the Japanse smelt eggs, we concluded that survival was very low at 24 hours or more of immersion in 90 and 100 g/L concentrations. Therefore, for those concentrations we used only the 6- and 12-hour intervals. Furthermore, the

145 solutions for 36-, 48-, 60- and 72-hour intervals were refreshed every 24 hours. Regarding pH
146 values, the ALC solution and dechlorinated water had 6.6 and 7.2 respectively, while the
147 cochineal dye solutions had between 7.1 and 7.2. After the marking procedure, the eggs were left
148 in incubators set at 12℃ until hatching. Water changes, removal of dead eggs and larvae, as well
149 as the recovery of hatched larvae were performed every 1 to 5 days, depending on their
150 conditions. The hatched larvae were cryopreserved in dechlorinated water at about -20℃.
151 Concerning survival evaluation, any egg that did not hatch within a week after observing the
152 majority of eggs in the same petri dish hatch was designated as dead. The survival rate was
153 calculated by using the following:

154 $$\text{Survival rate (\%)} = \text{number of hatched larvae} / \text{number of eggs} \times 100$$

155

156 *Confirmation and evaluation of markings*

157 Observation and photography of otoliths was carried out approximately 4 months after the
158 marking process, using a microscope (BX53) equipped with an epifluorescence system (BX3-
159 URA, U-LH100HG, BH2-RFL-T3) manufactured by the Olympus Corporation. The fluorescent
160 label of the otolith was confirmed at 100 magnification through a G-excited fluorescent mirror
161 unit (U-FGW: DM570, BP530-550, BA575IF), also manufactured by the Olympus Corporation.
162 The thawed larvae were lightly flattened with a cover glass so as not to break their otoliths,
163 where water was used as the cushioning substance. The fluorescence intensity was evaluated on a
164 4-point scale (1. invisible, 2. faintly visible, 3. visible, 4. visible brightly; Online Appendix 1)
165 with reference to Iglesias and Rodríguez-Ojea (1997). Mark quality evaluation was conducted
166 using 10 samples for each combination of concentration and immersion time. For this reason,
167 combinations with survival rates below 20 % were not evaluated as there were not enough
168 samples.

169

170 *Statistical analyses*

GLM (Generalized Linear Model; glm function; family: binominal) was used to evaluate the effects of concentration, immersion time and their interaction on the survival of Japanese smelt and the resulting mark quality. In order to use binominal distribution to evaluate the effects on mark quality, its four categories were integrated into two, wherein mark qualities of 1 (invisible) and 2 (faintly visible) were classified as unusable (1), while 3 (visible) and 4 (visible brightly) were classified as usable (2). The model was designed with reference to Sahashi and Morita (2017), and Miyamoto et al. (2021), and it goes as follows: logit (p) = $a1$ + $b1$ Concentration + $c1$ Immersion time + $d1$ ($b1$ Concentration × $c1$ Immersion time) with p = probability of survival or mark quality, $a1$ = regression constant, $b1$, $c1$, $d1$ = regression coefficient. To determine which of the four hypotheses mentioned in Introduction were relevant for this study, namely, how concentration and immersion time contribute to mark quality and survival, the 'dredge' function from the 'MuMIn' package, R (4. 0. 0), was used to test all possible combinations of the variables, including their interaction. The model selection for GLM was performed by the Akaike information criterion (AIC). The lower values of the AIC, which indicate the parsimonious model (Burnham et al., 2011), were used. The informative models were defined as models with delta AIC < 2.0 (Nemoto et al. 2018). The significance of the explanatory variables was evaluated using the likelihood ratio tests.

RESULTS

Examining the concentration / immersion data in aggregate, only 6% of the concentration / immersion combinations reached the mark quality of 3 and the survival rate of 60%. The best survival rate model has concentration and immersion time as significant factors, both individually and through their interaction (Table 1, Online Appendix 3). This model shows that there is a non-linear decreasing relationship between this interaction (g/L*h) and the survival rate, wherein at low concentrations, survival decreased with increasing immersion time but, at high concentrations, survival was relatively low at all immersion times (Table 1, Fig.1, Appendix

197 2). Based on the results of the survival rate experiment, combinations (100 g/L for all hours, 90
198 g/L for all hours, 80 g/L for 24-72 h, 70 g/L for 36-72 h, 60 g/L for 60-72 h, 50 g/L for 72 h and
199 40 g/L for 72 h) with survival rates below 20 % were excluded from the mark quality
200 experiment. The best models for explaining mark quality selected by AIC include concentration
201 and immersion time as significant variables (Table 2, Online Appendix 4). The results of
202 evaluating marked otoliths indicate that mark quality (1.0 = unusable, 2.0 = usable) improves
203 with increases in concentration and immersion time irrespective of the interaction between them
204 (Table 2, Fig.2).
205 Combining the results of the survival rate and mark quality experiments, the cochineal
206 dye marking conditions with mark quality of three or more and top three survival rates (65, 74
207 and 76 %) are 60 g/L for 24 h, 40 g/L for 48 h and 40 g/L for 36 h respectively (Fig. 3). On the
208 other hand, using 100 mg/L ALC had a survival rate of 83 % and in all cases the perfect mark
209 quality of four. The other control group (only water, no marking) had a survival rate of 86 % and
210 in all cases the mark quality of one.
211
212 **DISCUSSION**
213 In the current study, the interaction between concentration and immersion time
214 significantly contributed to survival. Therefore, it is important to focus on the combinations of
215 concentrations and immersion intervals within effective ranges for survivability. The reason why
216 the interaction between concentration and immersion time significantly contributed to survival
217 could be that the effect of high concentration is more detrimental than the effect of long-term
218 immersion (Online Appendix 2). One possible explanation is that the physiological state of
219 Japanese smelt eggs is sensitive to high cochineal dye concentrations. Meyer et al. (2012)
220 confirmed a similar tendency in the relationship between ALC concentrations and the hatching
221 success of the Baltic cod *Gadus morhua* embryos. Further study would be necessary to find the
222 cause of such a phenomenon, e.g. focusing on the individual components of the cochineal dye to

223 determine which ones are the most damaging for eggs. Additionally, the 86% survival for the
224 water control group suggests that a portion of the mortality was due to factors other than the dye
225 itself. These other factors may include the contamination of unfertilized eggs, the transportation
226 effect etc.

227 The results of evaluating marked otoliths indicate that mark quality improves with
228 increases in concentration and immersion time. Looking at the current study's mark quality and
229 survival rates, there seems to be a trade-off between mark quality and survival of Japanese smelt
230 when using a cochineal dye solution. Additionally, unlike survival rates, mark quality seems to
231 have been affected by both immersion time and concentration to a similar degree without the
232 interaction between them. Looking back at the four possible outcomes mentioned in the
233 Introduction of this paper, the results of this study preclude the first two hypotheses in which
234 neither or only one of the two factors (concentration and immersion time) is selected as
235 contributing to mark quality and survival. In the case of mark quality, the third hypothesis is
236 applicable, namely both factors contributed individually, while their interaction contributed to
237 survival, as indicated by the fourth hypothesis. These findings emphasize the need to focus on
238 both concentration and immersion time when determining the optimal marking conditions for
239 cochineal dye, not just for Japanese smelt, but possibly also for other freshwater fishes as well.

240 It is possible to use cochineal dye for marking eggs with acceptable results regarding the
241 clarity of the fluorescent otolith mark. Regarding the Japanese smelt hatchery industry, in general
242 terms, the minimum marking condition would be required to have a survival rate of above 60 %
243 and mark quality of three or more. Therefore, the results of this study suggest that the viable
244 marking conditions using cochineal dye on Japanese smelt are 40 g/L for 36 h, 40 g/L for 48 h
245 and 60 g/L for 24 h. Considering hatchery procedures, due to the time and costs required for the
246 management of water quality, space and electricity, there should be a preference for shorter
247 immersion intervals when developing a mass marking system. Therefore, in practical terms, the
248 optimal marking condition should be 60 g/L for 24 h.

249 The efficacy of ALC immersion-marking was tested by comparing the effects using
250 different ALC concentrations on the otolith of several fish species. For example, the optimal
251 treatment conditions for ayu *Plecoglossus altivelis* (Tsukamoto 1988) and Atlantic cod *Gadus*
252 *morhua* L (Blom *et al*. 1994) were 50-200 mg/L. However, only a few ALC studies have made a
253 comparison between different immersion periods. For Monteleone *et al*. (1993) claims that ALC
254 immersion periods of 6-24 h produce the best mark quality, survival and growth outcomes for
255 larvae. Most authors (e.g. Tsukamoto 1988; Blom et al. 1994), on the other hand, typically use
256 only the 24 h ALC immersion period. Regarding cochineal dye, it requires higher concentrations
257 and more immersion time than ALC treatments. According to Ohta (Kengo Ohta, Japan Patent
258 Kokai 2008-67648), usage of cochineal dye on fertilized eggs for otolith marking is generally
259 reported as follows: appropriate immersion time is around 24 h, while the concentration of the
260 cochineal dye is 2000 to 8000 mg/L for red sea bream *Pagrus major*, 2000 to 16000 mg/L for
261 devil stinger *Inimicus japonicus*, and 4000 to 16000 for bastard halibut *Paralichthys olivaceus*.
262 Therefore, when cochineal dye is part of a labeling agent or is an active ingredient of a chemical,
263 it can be widely used for marking fertilized eggs if the concentration is within the range of 4000
264 to 8000 mg/L, generally speaking. The current study suggests that the best 24-hour cochineal
265 solution for marking Japanese smelt otoliths is 60 g/L, which is 7.5 to 15 times that of the
266 aforementioned concentrations for sea fish. ALC otolith marking of rainbow trout is an example
267 of a reliable and cost-effective marking technique, wherein larvae are exposed to low
268 concentrations of ALC at higher pH values (8.8 - 8.9) (e.g. van der Walt and Faragher 2003).
269 Following this example, it might be possible to do the same with cochineal dye as well, namely
270 adjusting the pH of a cochineal dye solution to develop a marking technique that uses low
271 concentrations of cochineal dye.
272 In the current study, otolith marks were recognizable with no pre-treatment of the
273 otoliths required. It should be possible to estimate growth, survival and distribution patterns of
274 Japanese smelt for commercial interest, by conducting experimental releases of marked larvae

prior to recruitment, in the same manner as when using ALC. For example, Tsukamoto *et al.* (1989) estimated the abundance, dispersal and size-dependent survival of red sea bream *Pagrus major* juveniles that were marked by using ALC and released into a small bay (< 15 km^2). Secor and Houde (1995), performing a study on anadromous and marine species, reached the conclusion that ALC mark-release experiments were workable when larvae are released in closed systems. Based on the results of the previously mentioned studies, in conjunction with the findings of the current one, it would seem that cochineal dye can be an effective tool in marking Japanese smelt eggs. In real-life field study application, cochineal marking could be used, for example, for tracking hatchery fish in freshwater populations, as a way of assessing the effectiveness of a stocking program. For this reason, cochineal mark retention would be an important factor, as these types of field studies tend to last anywhere from a week to over a year. However, there are no freshwater fish studies using cochineal dye, making it difficult to speculate on how long a good cochineal mark (quality of 3 or 4) would be retained in a field study. Therefore, further study would be necessary to examine cochineal mark retention. Conversely, the current study is focused on the effects of cochineal dye marking on the survival and mark quality in freshwater fish larvae, not on the longevity of the applied marking. In that respect, it would also seem that it is less effective than ALC in relation to mark quality and survival. Additionally, the current study, mainly because of the extensive range of possible concentration / time combinations that needed to be examined, had only a single replicate to determine which of those combinations were optimal (Iglesias and Rodríguez-Ojea 1997, van der Walt and Faragher 2003). Therefore, to develop the best techniques in cochineal marking, it would be necessary to conduct studies with more replicates for greater precision and also evaluate the effects of more factors within the optimal range determined by the current study, for example, different water temperatures, different developmental stages of eggs etc.

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Compliance with ethical standards

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

Ethical approval Japan does not have regulations regarding experimentation on fishes, but the manipulation in the study on Japanese smelt was noninvasive and innocuous.

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418

Figure captions

Fig. 1 The relationship between: the number of survived eggs and cochineal concentration (g/L), immersion time (h) and interaction [cochineal concentration (g/L) × immersion time (h)]. The predictive lines are derived from a GLM analysis (Generalized Linear Model; glm function; family: binominal).

Fig. 2 The relationship between: mark quality (1.0 = unusable, 2.0 = usable) and cochineal concentration (g/L), immersion time (h) and interaction [cochineal concentration (g/L) × immersion time (h)]. The predictive lines are derived from a GLM analysis (Generalized Linear Model; glm function; family: binominal).

Fig. 3 The relationship between survival rates (%), mark quality, immersion time (h) and cochineal concentration (g/L). Open bar: survival rate; plot: mark quality. Error bars represent standard deviations. Mark quality data was not obtained for treatments when survival was less than 20%.