Title: A delayed effect of the aquatic parasite *Margaritifera laevis* on the growth of the salmonid host fish *Oncorhynchus masou masou*

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Abstract

Parasitic species often have detrimental effects on host growth and survival. The larvae of the genus *Margaritifera* (Bivalvia), called glochidia, are specialist parasites of salmonid fishes. Previous studies have reported negligible influences of the parasite on their salmonid hosts at natural infection levels. However, those studies focused mainly on their instantaneous effects (i.e., during the parasitic period). Given the time lag between physiological and somatic responses to pathogen infections, the effect of glochidial infection may become clearer during the post-parasitic period. Here, we examined whether the effect of glochidial infections of *Margaritifera laevis* on its salmonid host *Oncorhynchus masou masou* would emerge during the post-parasitic period. We performed a controlled aquarium experiment and monitored fish growth at two time intervals (i.e., parasitic and post-parasitic periods) to test this hypothesis. Consistent with previous observations, the effects of glochidial infection were unclear in the middle of the experiment (day 50; parasitic period). However, even with a natural glochidial load (48 glochidia per fish), we found a significant reduction in growth rates of infected fish in the extended period of the experiment (day 70; post-parasitic period). Our results suggest that examining only instantaneous effects may provide misleading conclusions about mussel–host relationships.

Key words: Host–parasite interactions, freshwater mussels, salmon, glochidia, growth rate

Introduction

In freshwater ecosystems, enigmatic epidemics of harmful parasites have occurred over the past few decades, representing serious threats to freshwater biodiversity (Daszak et al. 2000; Pounds et al. 2006; Rohr et al. 2008). In some cases, parasites trigger the population collapse of freshwater fishery resources and can have an important burden on global economy (Krkošek et al. 2005; Costello 2006; Krkošek et al. 2006; Krkošek et al. 2007). Therefore, understanding the impacts of parasites on host species is a critical issue in freshwater ecology.

Notable examples have revealed the population-level consequences of parasites for host species (Pounds et al. 2006; Krkošek et al. 2007; Rohr et al. 2008). However, at an individual level, empirical evidence for parasite-induced reductions in body condition and/or survival rates are often limited to heavily infected hosts (Ferguson et al. 2011; Taeubert and Geist 2013; Lhorente et al. 2014; Mayo-Hernandez et al. 2015; Raffel et al. 2015; Filipsson et al. 2016). This gap is problematic because failure to detect true impacts of parasites may lead to an overly optimistic assessment of parasite threats. As one potential mechanism filling this gap, we suggest that the negative effects of parasites on host performance (e.g., growth) may accumulate and become apparent through time. Most studies focused on the instantaneous effects of parasites and dismissed potential time delays of host species responses. Immune responses are associated with physiological costs, but would require some time to impact

somatic growth given the slow turnover rates of body tissues (Harvey et al. 2002; McIntyre and Flecker 2006). Thus, we hypothesized that the negative effects of parasite infection on host performance may be initially "cryptic," emerging clearly during the late- or post-parasitic period.

The freshwater pearl mussel *Margaritifera laevis* (family Margaritiferidae) inhabits cool-water streams (temperature usually < 20°C) in Japan (Kondo 2008). The larvae of *M. laevis*, called glochidia, are specialist parasites of masu salmon *Oncorhynchus masou masou* and *O. m. ishikawae* (Kondo 2008). This parasite species serves as an excellent model organism to test our hypothesis for the following reasons. First, the mussel species employs a simple infection strategy in which female mussels release glochidia into the water column and parasitize the gills of masu salmon (Kondo 2008; Haag 2012). This simplicity in the infection process allows us to control glochidial infection experimentally. Second, their parasitic stage is known to be ca. 40–50 days (Kondo 2008). This provides us an essential piece of information for clarifying "parasitic" and "post-parasitic" periods in an experiment.

The impacts of glochidia at natural infection levels may have been underestimated in the previous studies as they focused mainly on instantaneous effects on host growth or survival (Treasurer and Turnbull 2000; Treasurer et al. 2006; Taeubert and Geist 2013). There is a certain possibility that glochidial impacts may emerge during the post-parasitic period given the slow

turnover rates of somatic tissues in fish species (Harvey et al. 2002; McIntyre and Flecker 2006). Here, we examined the effects of glochidial infection on host growth at two time intervals (i.e., the parasitic and post-parasitic periods) by artificially infecting *O. m. masou* host fish with *M. laevis* glochidia. We performed this experiment in a controlled aquarium setting, which allowed us to evaluate any delayed effects of the parasite.

Method

Glochidia infestation

Gravid female mussels were collected from the Chitose River on 16 July, 2015 to create an infestation bath. We selected one gravid female (shell length: 85 mm, wet mass: 52.8 g) to avoid any confounding effects due to the glochidia source. We created a 10-L infestation tank with a glochidial density of 40,000 glochidia L^{-1}. Another bath with the same volume but no glochidia was used as a control tank.

We obtained 75 masu salmon individuals from the Salmon and Freshwater Fisheries Research Institute, Hokkaido Research Organization, Japan, where the species has been reared for multiple generations. We did not use wild individuals from the Chitose River, since previously infected fish may exhibit acquired immune response to glochidial infection

88 (Treasurer et al. 2006). Average initial fork length (FL; mean ± standard deviation) and body
89 weight (wet mass) were 71.8 ± 1.4 mm and 3.8 ± 0.4 g, respectively. The body size was
90 intended to reflect the range of FL in natural conditions (Terui et al. 2014; Terui and Miyazaki
91 2015). In total, 45 individuals were placed in the infestation tank for 30-min, while the
92 remaining 30 individuals were kept in the control tank at the same time. Fifteen infested
93 individuals were chosen randomly and sacrificed to measure glochidial load. The average
94 glochidial load per fish was 844 ± 184 (n = 5) 30 min after infestation but decreased to 48 ± 29
95 after 10 days (n = 10). This glochidial load was comparable to field observations from the
96 Chitose River, Hokkaido, Japan (see Fig. S1 in Appendix). The remaining 60 fish were used in
97 the experiment described below.
98
99 *Experimental design*
100 The laboratory experiment was carried out from 17 July to 24 September 2016, using 10
101 experimental aquariums (60 cm × 30 cm × 15 cm) with a built-in flowthrough system at the
102 Salmon and Freshwater Fisheries Research Institute. The experimental period was designed to
103 be consistent with the parasitic period of *M. laevis* in natural environments (Kondo 2008; Terui
104 et al. 2014; Terui and Miyazaki 2015). River water (the origin of the river water has no mussel)
105 was continuously trickled from the top of each aquarium using PVC pipes (diameter: 30 mm,

length: 400 mm) with valves to maintain water quality, ambient oxygen levels, and water temperature. The water temperature was 9.4 ± 0.3˚C (range: 8.9–10.7 ˚C) as measured with an automated HOBO® Pendant Temperature/Light 8K Data Logger (Onset Computer Corp., Bourne, MA, USA). The light regimen mimicked natural light conditions.

Each treatment and control aquarium had 5 independent replicates (10 aquariums total). Treatment and control aquariums were assigned randomly. On 17 July (day 0), we released six infected fish (treatment) or six uninfected fish (control) into each aquarium. All fish had been fasted for 24 h before the experiment and were batch-marked with fluorescent visible implant elastomer (VIE) tags (six colors; Northwest Marine Technologies, Shaw Island, WA, USA) applied to the adipose tissue behind the nose. We fed the fish artificial food items (EX masu No.4; Nosan Corp., Yokohama, Japan), which were provided at the river water inflow 13 times per day (daytime only) using autofeeders. The total daily ration was roughly 5% fish body mass. We aerated each aquarium with an air-stone at the outlet but used no substrates to avoid any confounding effects. No mortality was observed during the experimental period.

This experimental design mimics two important aspects of natural conditions. First, although the prevalence of glochidia on masu salmon is extremely high near mussel beds (~100%), infections rarely occur in no-mussel areas (Terui and Miyazaki 2015). Thus, the experimental setting of the treatment (100% prevalence) and control groups (0% prevalence)

was appropriate. Second, masu salmon is exposed usually to intraspecific competition in natural environments (Nakano 1995). Thus, the impacts of glochidia infection should be evaluated under the influence of intraspecific competition. Although the fish density in our experiment was very high (6 ind./0.18 m^2), preliminary observations suggested that this setup is required to motivate among-individuals competition in our experiment (personal observations, KO and HU).

The change in body weight over time was used as the growth rate for the experiment. We measured the wet mass of fish on days 0 (initial day), 50 (middle), and 70 (end of experiment).We considered day 50 to be the end of the parasitic period, because *M. laevis* glochidia requires 50 days for metamorphosing into juvenile mussels under the water temperature setting in our experiment (~13 °C; Kobayashi and Kondo 2005; Kondo 2008). Accordingly, day 70 was referred to as the post-parasitic period. After carefully shaking off water droplets, FL (to the nearest mm) and wet mass (± 0.1 g) of each fish were measured. The initial FLs and wet mass were not significantly different between the treatment and control groups (infected fish: 72.0 ± 1.4 mm and 3.9 ± 0.4 g, uninfected fish: 71.5 ± 1.3 mm and 3.8 ± 0.3 g; t-test, p-value = 0.23 for both). Also, there was little variation in mean FL and wet mass among aquariums (range: 70.0–72.8 mm in FL, 3.6–4.0 g in wet mass), although one pair of aquariums had significant difference in FL (pairwise t-test, p-values were > 0.2 for all

combinations after Holm correction, except one pair that had a value of 0.02). We estimated the specific growth rates (SGRs) based on the following equation (cf. Matsuzaki et al. 2012; Hasegawa et al. 2014):

$$SGR = 100*(\ln W_{end} - \ln W_{initial})/t \quad \text{eq. 1}$$

where W_{end} and $W_{initial}$ are the fish wet mass on the final (day 50 or 70) and initial (day 0) days of the experimental period, respectively, and *t* is the time elapsed between the measurements (day). Delayed effects of glochidial infection might be quantified through SGRs in the period of day 50–70. However, we believe that this measure is not appropriate here because this may overlook any "cumulative" effects of glochidial infection on SGR.

We also calculated Fulton's condition factor (K):

$$K = (W_{initial}*100)/FL^3 \quad \text{eq. 2}$$

where W and FL are the wet mass (g) and FL (cm) of each fish individual, respectively. This provided a quantitative measure of fish body condition. We preliminary confirmed that initial body condition, rather than initial FL or wet mass, was a good variable for predicting final

dominance status in the aquarium (Fig S2, Appendix) and assumed that the variable would explain some variation in fish growth rates (see *Statistical analysis*). The initial *K* was not significantly different between the treatment and control groups (infected fish: 1.0 ± 0.1, uninfected fish: 1.0 ± 0.1; t-test, p-value = 0.55) as well as among aquariums (pairwise t-test, p-value = 1.0 for all combinations after Holm correction).

Statistical analyses

To examine the effects of glochidial infection on SGR (days 50 and 70), we employed a hierarchical Bayesian model. We assumed that SGRs of individual fish *i* in aquarium *j* followed a normal distribution, $SGR_i \sim Normal(\mu_i, \sigma_1^2)$, and that the mean μ_i was related to linear predictors with an identity-link function as follows:

$$\mu_i = \alpha_{j(i)} + \beta 1 * K_i + \varepsilon_{1i} \quad \text{eq. 3}$$

where $\alpha_{j(i)}$ is an aquarium-specific intercept and β is the regression coefficient of Fulton's condition factor at the start of experiment. The Fulton's condition factor *K* was standardized with a mean 0 and SD 1 prior to the analysis. Note that we included the Fulton's condition factor *K* as a predictor to control its potential effects on SGR, which could otherwise lead to

biased estimates of the infection treatment effect. The term ε_{1i} is the residual error at the individual level that was governed by the variance parameter σ_1^2. The aquarium-specific intercept was modeled as follows:

$$\alpha_j = \alpha_{global} + \beta 2 * Treatment_j + \varepsilon_{2j} \quad \text{eq. 4}$$

α_{global} is a global intercept and $\beta 2$ is the effect of infection treatment. The variable $Treatment_j$ was expressed as a dummy variable (infection = 1, control = 0). Another term ε_{2j} is the residual error at the aquarium level and was normally distributed as $\varepsilon_{2j} \sim \text{Normal}(0, \sigma_2^2)$. This hierarchical formulation allowed us to extract the treatment effect independent of random variations among aquariums; i.e., a group of six fish was treated as one replicate when examining the effect of the infection treatment. Without the hierarchical structure of the model (e.g., ANCOVA), the treatment effect $\beta 2$ confounds with the random effect (i.e., random variation among aquariums). Our preliminary analysis revealed no effect of an interaction term between condition factor and infection treatment (linear mixed effect model, p-value > 0.5 for both time intervals). Therefore, we did not consider the interaction term further in the analysis.

The parameters were assigned vague priors; i.e., normal distributions (mean = 0, variance = 10^4) for α_{global}, $\beta 1$, and $\beta 2$, and gamma distributions (mean = 1, variance = 10^4) for

196 the inverses of σ_1^2 and σ_2^2. The model was fitted to the data with JAGS (ver. 4.1.0) and the
197 package "*rjags*" in R 3.2.3 (Plummer 2014). Three Markov Chain Monte Carlo chains were run
198 with 7,500 iterations (2,500 burn-in) and 500 samples per chain were used to calculate posterior
199 probabilities. Convergence was assessed by examining whether the R-hat indicator of each
200 parameter approached 1 (Gelman and Hill 2007).
201
202
203 **Results**
204 Although wet mass was little varied among individuals at the start of the experiment, the final
205 wet mass and SGRs at days 50 and 70 differed greatly (Fig. 1). In the control aquariums,
206 individuals grew consistently throughout the experimental period, gaining larger body sizes and
207 wet mass through time (mean FL = 87.3 ± 6.9 mm at day 50, 93.6 ± 6.6 mm at day 70; mean
208 wet mass = 7.3 ± 1.8 g at day 50, 8.6 ± 2.1 g at day 70). In contrast, infected individuals grew
209 slowly and seemed to stop growing during the post-parasitic period (mean FL = 84.4 ± 7.5 mm
210 at day 50, 86.4 ± 7.8 mm at day 70; mean wet mass = 6.3 ± 1.9 g at day 50, 6.8 ± 2.1 g at day
211 70). The histograms of FL and wet mass for infected individuals largely overlapped at day 50
212 and 70 (Fig. 1).
213 The Bayesian model revealed delayed responses of host fish to glochidial infection. At

214 the aquarium level, fish in the infection treatment did not have reduced growth rates in the
215 middle of the experiment (i.e., day 50; Table 1 and Fig. 2). However, the effect of infection on
216 SGR became evident by the end of the experiment (i.e., day 70; Table 1 and Fig. 2). At the
217 individual level, condition factor *K* had a marginal influence on SGR in both time intervals
218 (days 50 and 70) (Table 1). The variance at the individual level (σ_1^2) ranged from 0.37 (day 70)
219 to 0.47 (day 50), while the aquarium-level variance (σ_2^2) ranged from 0.06 (day 70) to 0.20 (day
220 50).
221
222
223 **Discussion**
224 Earlier studies have reported insignificant influences of glochidial infections on host growth at
225 natural infection levels (Treasurer and Turnbull 2000; Treasurer et al. 2006). The results of the
226 present study were consistent with these observations, as the effect of glochidial infection was
227 unclear in the middle of the experiment (day 50). However, even at natural glochidial load (48
228 glochidia per fish on average), the growth rate of the infected fish group was reduced
229 significantly during the extended period of the experiment (day 70; post-parasitic period). These
230 results suggest that examining only instantaneous effects may result in misleading conclusions
231 about mussel–host relationships.

232 The delayed response of host growth may reflect the fact that somatic tissue growth
233 lags behind physiological responses to parasite infection. It is well known that salmonid fishes
234 have quick immune responses to pathogenic infections (Collet 2014). Similarly, Thomas et al.
235 (2014) reported a rapid rise in ventilation rate in salmonid fishes immediately following
236 glochidial infection. However, turnover rates of body tissues seem to be much slower than
237 physiological responses. For example, nitrogen in the muscle tissues of lake trout has a half-life
238 (i.e., 50% replacement) of 69 days (Harvey et al. 2002; McIntyre and Flecker 2006). Although
239 nitrogen turnover should not be interpreted as a direct measure of body tissue turnover rates, it
240 still suggests that somatic growth does not immediately reflect the physiological costs
241 associated with immune responses in salmonid hosts. Alternatively, the delayed effects of
242 glochidial infection could be attributable to coinfection of other diseases (e.g., Lhorente et al.
243 2014). However, in our controlled experimental conditions, it is unlikely that other infectious
244 diseases had thrived and impacted on salmonid fish growth. Therefore, the time lag between
245 physiological and somatic responses is likely to explain the observed delayed effects of
246 glochidial infection.
247 In our study, the effect of initial body condition on host SGR was not significant.
248 Salmonid fish with better body conditions may have a high competitive ability, allowing them to
249 grow faster than subordinate individuals (Nakano 1995; Yamamoto et al. 1998). Indeed, we

found a positive relationship between initial condition factor and final dominance status (Fig. S2, Appendix). However, we confined the variation of initial body condition among host fish to focus on the main effects of parasites on host growth. Thus, the slight difference of body condition in our experiment may be insufficient to detect any effects of body condition on host growth. In the future studies, however, potential interactions between body condition (e.g., body size) and infection status should deserve further attention. For example, earlier studies have reported decreased foraging activity and/or dominance performance among heavily infected fish (Österling et al. 2014; Filipsson et al. 2016). This may in turn cause a relaxation of competition among heavily infected fish hosts. Meanwhile, intraspecific competition could be intensified if glochidial infection reduce disproportionately competitive ability of subordinate individuals (cf. Hatcher et al. 2006).

Our results should be viewed with some caution. First, glochidia used in our experiment were derived from one gravid female mussel. Since there are many sources of glochidia in natural environments, this experimental setup may fail to reflect the natural variation in the virulence of glochidia. Second, the host fish density in our experiment is very high given the masu salmon density in natural streams (typically < 0.5 individuals per square meter; cf. Inoue et al. 1997). Although our experimental fish (captive fish) seemed to have adopted to the high density environment, such a condition may result in reduced growth

performance in general (Gilmour et al. 2005; Fernandes et al. 2015). Hence, our results should be interpreted carefully when comparing with the data from natural environments.

Although there are several limitations, this study confirms a fundamental phenomenon that has been overlooked in mussel–host relationships: the delayed effect of glochidial infection. Delayed effects of parasite infection have been less appreciated in the literature, but this phenomenon could be widely applicable to other host-parasite interactions given the presumed underlying mechanism (the time lag in physiological and somatic responses to parasite infection). Further studies should explore whether this delayed effect has substantial impacts on host survival and life-history traits in the long term. Such information may provide critical insights towards understanding host-parasite relationships in aquatic ecosystems.

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369

370

Table 1 Estimated parameters in the hierarchical Bayesian model that explains SGRs. Estimates whose 95% credible intervals (CIs) did not include zero are shown in bold.

Duration	Effect	Estimate	95%CI	
			Low	High
Day 50	Intercept (α_{global})	**1.27**	0.99	1.56
	Condition factor (β1)	0.10	−0.03	0.22
	Infection (β2)	−0.39	−0.80	0.01
Day 70	Intercept (α_{global})	**1.15**	1.00	1.32
	Condition factor (β1)	0.07	−0.03	0.16
	Infection (β2)	**−0.42**	−0.64	−0.21

Figure caption

Fig. 1 Histograms of fork length (top) and wet mass (bottom) at day 0, 50 and 70. Left and right panels show control (uninfected) and treatment (infected) groups, respectively. Different colors indicate different time intervals (white: day0, gray: day 50, red: day 70).

Fig. 2 Influence of glochidial infection on the growth (specific growth rate [SGR] in wet mass) of the host fish, *Oncorhynchus masou masou*, along a gradient of initial Fulton's condition factor (standardized to a mean 0 and SD 1). White and black dots denote uninfected and infected host fish, respectively. Fitted lines (white, uninfected; black, infected) were derived from the hierarchical Bayesian model. Gray shaded areas are 95% CIs of prediction (dark gray, uninfected; light gray, infected).

Figure 1 Ooue et al.

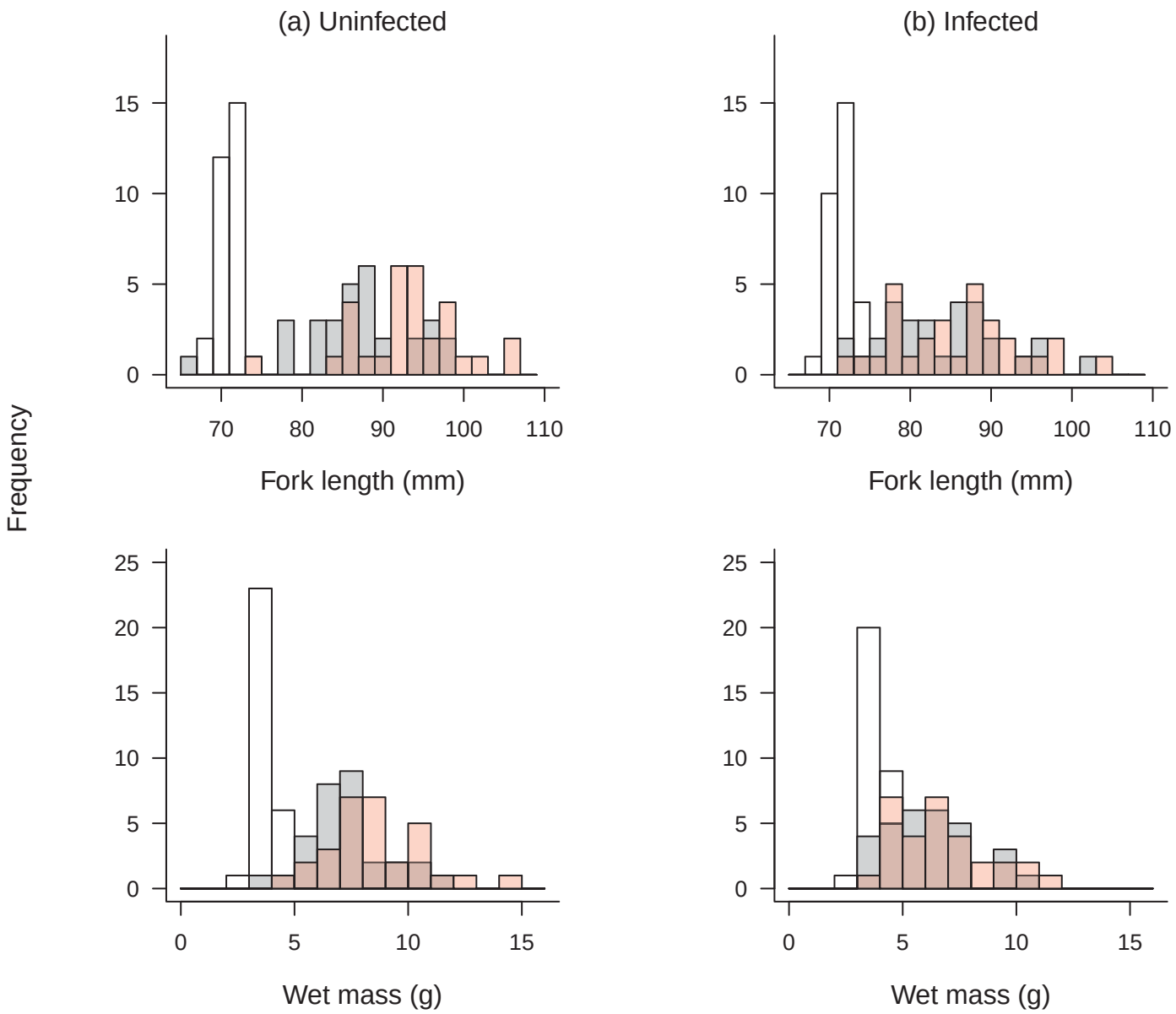

Figure 2 Ooue et al.