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Title: Testing local adaptations of affiliate freshwater pearl mussel, *Margaritifera laevis*, to its host fish, *Oncorhynchus masou masou*

Hitoshi Kitaichi[1], Junjiro N. Negishi[2]*, Daisetsu Ito[1], Kazuki Miura[1], Hirokazu Urabe[3]

[1] Graduate school, Hokkaido University, Sapporo 060‐0810 Japan

[2] Faculty of Environmental Earth Science, Hokkaido University, Sapporo 060‐0810 Japan

[3] Salmon and Freshwater Research Institute, Hokkaido Research Organization, Eniwa 061-1433 Japan

Contact e-mail addresses for authors:
H Kitaichi: h.northcity@gmail.com
JN Negishi: negishi@ees.hokudai.ac.jp
D Ito: kamuymintara_chirai@yahoo.co.jp
K Miura: kmiura8@gmail.com
H Urabe: urabe-hirokazu@hro.or.jp

*Corresponding author

Abstract

Understanding the limiting factors of the reproduction process in host–affiliate relationships is a high priority. We examined the effects of habitat location on the reproductive process of freshwater pearl mussels *Margaritifera laevis* (Bivalvia, Unionida) as a parasite using sympatric and allopatric *Oncorhynchus masou masou* (Actinopterygii, Salmoniformes) as a host fish. Initial infection rates of parasitic larvae (glochidia) and transformation rates to cysts (encysted glochidia) were examined for all parasite-host combinations from three habitat locations (a total of nine combinations) to test the hypothesis that sympatric pairs of mussels and fish result in the highest success rates of glochidia infection and encystment. Measurements of glochidia-infected fish reared in flow-through experimental indoor tanks were taken at the initial infection point as well as at encystment, two weeks after the infection. Results disagreed with our hypothesis. Instead, an unexpected heterogeneity in a pathological deformity in gills explained a greater amount of variance in these processes. This deformity was responsible for reducing the initial infection rate and increasing the metamorphosis rates of initially attached glochidia to cysts. The field-measured prevalence of the gill deformity was low in all habitat locations, indicating that the deformity occurred during the acclimation period before infection for relatively small-sized host fish more susceptible to infection. Our results did not show the local adaptation of parasitic freshwater mussels to host fish but shed light on one of the least studied factors, providing an empirical underpinning of the importance of pathologically diversified host conditions in the reproductive processes of unionid mussels.

Keywords: endangered species; host-parasite; immunity; infection; parasites

Introduction

Organisms are selected to maximize reproductive success, and thus fitness (i.e., number of surviving offspring) within a variety of constraints imposed by phylogeny, development, genetics, and stochastic environments. Success is a function of the reproductive strategy of organisms, and elucidations of successful patterns and mechanisms is of paramount importance for an improved understanding of the population dynamics of organisms (Fleming, 1996; Judson, 1994). Organisms that possess relatively complex life histories in their reproductive stages, such as parasites, parasitoids, and symbiotic-hosts (*sensu* host–affiliate relationships after Koh et al., 2004), play significant roles in the functioning and services of ecosystems (Hudson et al., 2006; Lafferty et al.,2008). Affiliate organisms are disproportionately exposed to an increasing number of threats deriving from external environmental changes such as climate change and habitat loss (Colwell, Dunn, & Woolnough, 2012). Understanding any factors which limit the reproductive process in host–affiliate relationships is needed to promote conservation on them.

Freshwater mussels (Order: Unionida) are an example of an aquatic affiliate species whose complex reproductive strategy is dependent on fish. They are parasitic organisms whose larvae (glochidia) require temporary attachment to the tissue of the appropriate host fish (Bauer & Wächtler, 2012; Strayer, 2008). Glochidia become encysted by the migration of host cells and/or the thickening of gill tissue when attached to a compatible host (Nezlin et al., 1994; Rogers-Lowery & Dimock, 2006). Successful glochidia remain encysted for days to months, depending on the species as well as

74 external environmental factors such as temperature (Huber & Geist, 2017; Marwaha et
75 al., 2017; Roberts & Barnhart, 1999; Ziuganov et al., 1994). When their development is
76 complete, glochidia transform into juvenile mussels (i.e., metamorphosis) and drop off
77 from the host to initiate their juvenile life stage in the bed substrate (Negishi et al.,
78 2018; Wächtler, Dreher-Mansur, & Richter, 2001). Specialist unionids are host-specific
79 and have a limited number of suitable host fish, whereas other generalists with lower
80 host specificity are compatible with a wider range of fish species (Haag, 2012; Huber &
81 Geist, 2017; Wacker et al., 2019). Their relatively sessile nature coupled with a complex
82 parasitic life cycle is a key feature of their vulnerability to various kinds of human
83 pressures (Modesto et al., 2018). Consequently, they are among the most threatened
84 group of organisms globally and more research is needed for their conservation
85 (Ferreira-Rodríguez et al., 2019; Haag, 2012; Negishi et al., 2008).
86 Many intrinsic and extrinsic factors affect the success rate of attachment and
87 transformation of glochidia to cysts and then their eventual metamorphosis into juvenile
88 mussels. Host compatibility (i.e., the proportion of successfully metamorphosed
89 juveniles from initially attached glochidia) could vary for multiple reasons. The history
90 of infections can affect transformation success because the host fish may acquire post-
91 natal immune resistance after multiple infections (Rogers & Dimock, 2003). The stress
92 level and body condition of the host may also be important (Douda et al., 2018). The
93 effectiveness of natal immunity is affected by ambient temperature which can indirectly
94 mediate temperature-dependent immunity response (Roberts & Barnhart, 1999;
95 Taeubert, El-Nobi, & Geist, 2014). Recent studies have increasingly recognized the

importance of the control of population-level evolution of parasitic interactions between sympatric host fish and mussels (Caldwell et al., 2016; Douda et al., 2014; Rogers, Watson, & Neves, 2001; Salonen et al., 2017; Taeubert et al., 2010, 2012; Wacker et al., 2019). An important evolutionary process behind population-specific host-parasite relationships is local adaptation, in which natural selection increases the frequency of traits of hosts or parasites within a population that enhance the survival or reproductive success of the individuals expressing them (Greischar & Koskella, 2007; Morgan et al., 2005; Taylor, 1991). With many experimental studies available on local adaptation in host-parasite relationships (Greischar & Koskella, 2007), freshwater mussels have also been used as a model organism to test local adaptation (Douda et al., 2017). They provide a unique case where the host has a far lower life span than the mussel parasite (Bauer, 1997; Taeubert & Geist, 2017).

Freshwater pearl mussels belonging to the family Margaritiferidae inhabit cold waters, are generally long-lived, and provide various ecosystem functions (Howard & Cuffey, 2006; Limm & Power, 2011; Ziuganov et al., 2000). Pearl mussels are generally host-specific and parasitize the gills of a limited numbers of host species (Kobayashi & Kondo, 2009; Lopez et al., 2007; Taeubert et al., 2010). The imperiled population status of this group across its geographical range has increased global efforts to conduct ecological research to better protect and restore their habitat and to actively assist their reproduction (Araujo & Ramos, 2000; Bolland et al., 2010; Geist, 2010; Österling, Arvidsson, & Greenberg, 2010). *Margaritifera laevis* is distributed in Sakhalin, Russia, and Honshu and Hokkaido islands of Japan, and is an obligate parasite that requires

Oncorhynchus masou masou (*O. masou ishikawae* in western Japan) as a compatible host (Kondo, 2008). Like other species in this family, *M. laevis* has been designated as a high conservation priority with the status of “endangered” in the Red List of Japan (Akiyama, 2007; Miura et al., 2019). Understanding of population-specific host-parasite relationship helps to clarify the spatial scales of ecosystem management units (Österling, Larsen, & Arbidddon, 2020). However, no studies have rigorously examined the host-parasite relationship of *M. laevis* in the context of local adaptation.

Taeubert and Geist (2017) proposed several possibilities on the patterns of local adaptation for a species in the same genus, *Margaritifera margaritifera*, and stated the absence of empirical data supportive of the local adaptation of parasite to host (i.e., higher infectivity in sympatric pairs compared to allopatric pairs). However, there have been few attempts using a full factorial experimental design involving multiple populations of unionoid mussels and host fish (Schneider et al., 2017), which is an ideal study setup to address this question (Kawecki & Ebert, 2004). Furthermore, Akiyama (2007) preliminarily reported on the possibility of sympatric host-parasite pairs as a cause of high survival of metamorphosed juveniles of *M. laevis* and thus the presence of local adaptation of mussels to host fish. In this study we report the results of a controlled laboratory experiment in which the reproductive process of *M. laevis* was examined using pairs of sympatric and allopatric combinations with its host fish, *O. masou masou*. We focused on two critical life-cycle stages in their reproduction, initial glochidia infection and transformation to cysts (encystment). We hypothesized that the success rates of these stages are affected by local adaptation; whether the host fish and

mussels are of sympatric origins or not was expected to make a difference in the outcome. We specifically predicted that sympatric pairs (collected from the same locality) would show the highest rates of initial infection and encystment. We also examined the effects of gill conditions on these rates because preliminary examinations of sacrificed fish samples revealed that some fish possessed a symptom of a pathologically induced abnormal gill morphology. We therefore predicted that both rates would be higher in the affected individuals compared to those without symptoms because of reduced immunity. Lastly, the occurrence of such a gill abnormality was assessed for field-collected individuals to determine if the symptom was persistent in the field.

Methods

Study sites

We collected mussels (*M. laevis*) and fish (*O. masou masou*) for the experiment from three rivers (Abira River, Chitose River, and Shakoton River) where both species occur sympatrically (Fig. S1). All the rivers were forested in the riparian zones and flow into the sea (Abira River to the Pacific Ocean and two others into the Sea of Japan). Rivers are isolated from each other by the sea. Some *O. masou masou* descend to the sea with their sea lifecycle continuing for 2.0 to 3.5 years. Because it is unlikely that the attached mussel glochidia from one river survive through this period and grow as juveniles, we assumed that currently there is no gene flow among freshwater mussels in these rivers. The Chitose River is larger (approximately 10 m wide) in terms of channel wetted width

compared to two others (<5 m). The catchment area of Chitose River is 1244 km^2, followed by 539.2 km^2 (Abira River) and 75.6 km^2 (Shakotan River). The Chitose River begins as an outlet of Lake Shikotsu whereas the other two originate from mountains. Because of this, the Chitose River is characterized as having a more stable flow rate compared to the two other rivers. No records of transplanting mussels (*M. laevis*) exists whereas there were some records of stocking young-of-the-year juvenile fish (*O. masou masou*) in the Chitose River and Shakotan River according to unpublished data from Hokkaido National Fisheries Research Institute and Salmon and Freshwater Fisheries Research Institute (H Urabe, personal correspondence). Approximately 0.2 million and 1.3 million *O. masou masou* were stocked from other rivers in the Chitose River (over the period 1955–2018) and Shakotan River (over the period 1981–1987), respectively. The stocking in Shakotan River was possibly substantial enough to affect genetic structure of native fish population because of relatively small, estimated population size of native fish to the stocked fish. However, recent genetic analyses using microsatellite markers showed that genetic structure of native fish population remains having characteristics undistinguishable from those in nearby rivers without fish stocking (H Urabe, unpublished data).

We used nine water tanks (30 cm × 60 cm × 30 cm) held in the Hokkaido Research Organization facility in Eniwa City (Figs. S1 & S2). These indoor tanks had a flow-through system and were provided with a continuous flow of fresh water directly diverted from the Kashiwagi River nearby; temperature and electrical conductivity of inflowing water ranged from 13.3–13.8°C and 8.41–8.53 mS/m, respectively. Lighting

was provided with ordinary fluorescent lamps on the ceiling and the duration was adjusted to diurnal cycles (10-h dark to 14-h bright cycles).

Field sampling for experiments

Young-of-the-year juveniles of *O. masou masou* were collected using an electrofisher (Model 12, Smith-Root Inc., Vancouver, WA, USA) and scoop nets on June 6, 15, 19, 2017 (Abira River, 243 individuals), June 13, 2017 (Chitose River, 271 individuals), and June 7, 14, 2017 (Shakotan River, 231 individuals). Immediately after the fish were caught, fish were transported to the facility in an iced, well-aerated container, and kept in the experimental tanks until the experiment. Fish were fed with commercially available dry pellet feeds eight times per day with automated feeders (model: NAT-108). This feeding protocol was maintained throughout the experiment. On June 23, 2018, each collection site was revisited, and we collected at least 10 young-of-the-year juveniles of *O. masou masou* by electrofishing. These additional fish were used to check gill conditions in the field. Preliminary examinations of these fish showed that they were not infested with mussel glochidia. We used young-of-the-year fish and thus we considered that these fish did not have previous contact with *M. laevis* glochidia.

We collected gravid mussels for the experiment on July 22, 2017 in the Abira River, on July 28, 2017 in the Chitose River, and on July 30, 2017 in the Shakotan River. The species is known to reach a peak of maturation around this time of the year (Akiyama, 2007). We checked gravidity *in situ* by carefully prying open the shells, extracting a small amount of glochidia, and checking under a stereoscopic microscope

for their activity levels. We used a small amount of NaCl to check the valve closure as an indication of their viability. Approximately 20 individuals with indications of matured glochidia were collected from each river and immediately transported to the facility. To minimize potential variations in glochidia condition related to mussel size, ten similar -sized individuals were selected, placed in 2-liter pales filled with the river water (500 ml), and agitated strongly by aerating the water for 30 minutes. Five individuals for the Abira River (shell size range: 105.0–122.0 mm), three individuals for the Chitose River (80.5–97.0 mm), and five individuals for the Shakotan River (98.0–110.0 mm) ejected glochidia and thus were used in the experiment. Water containing ejected live active glochidia (i.e., glochidia water) was pooled for each river used for the infection treatment process.

Infecting host fish with glochidia and rearing

We exposed host fish to mussels on the same day when corresponding gravid mussels were collected from rivers. We prepared three subsets of individuals (61 individuals each) from fish collected in each of the three rivers (hereafter referred to as fish strain). Each of the three subsets of fish was infected with mussel glochidia from the three rivers (hereafter referred to as mussel strain), resulting in a factorial design with nine treatments (a total of 549 fish were used; Fig. S3). Each subset of fish was immersed in 20 L of well-aerated water that was treated with glochidia water for 32 minutes. We examined the preliminarily concentrations of glochidia in well-stirred glochidia water and diluted with river water to adjust the glochidia concentration, ensuring

concentrations were below those lethal to host fish (40,000 glochidia L^{-1}; Ooue et al., 2017). The final concentration of glochidia during infection was estimated by collecting 250 ml of water immediately after the infection (one each from the respective tanks), preserving the sample with 50% ethanol, and taking averages of the counts of glochidia in 10-time replicated 200 micro litter subsamples under microscopes (Table 1). Infected fish subsets were returned within an hour to each of nine flow-through tanks. Before this return, five infected host fish were randomly collected from each subset, sacrificed, and preserved in 10% formaldehyde solution (samples for initial infection).

Measurements

Attached glochidia of *Margaritifera* typically become completely encysted within several days once the initial attachment occurs successfully (Araujo et al., 2002; Soler et al., 2018; Ziuganov et al., 1994). Thus, in addition to the samples for initial infection, which were collected within an hour of the infection, we collected another set of five randomly selected individuals from each tank at 16 or 17 days post-infection (samples for encystment) and preserved them in 10% formaldehyde solution. All the fish samples (N=90) were measured for their fork length (mm), standard length (mm), and wet body mass (g). Infection rates were measured by removing four pairs of gills from each fish (a total of eight for each) and counting the number of glochidia or cysts on each. In this process, we noticed that gill filaments were inflated abnormally with partial conglutination of gill filaments and lamella in some individuals (Fig. S4). Thus, we visually recorded the gill conditions of glochidia (or cyst) attachments, noting whether

gill filaments were normal or deformed. Digital images of each gill were obtained by a stereoscopic microscope (equipped with an Olympus DP20 digital camera). These images were later processed using an image-analyzing software ImageJ (Schneider, Rasband, & Eliceiri, 2012) to quantify the total area as well as the deformed area of filaments. This image analyses were conducted only on gills of samples from the initial infection; no analyses were done on samples at the encystment stage because we assumed that total area as well as deformed/normal areas of filaments would not change substantially during the experiments.

Analyses

The following analyses were performed using R (Version 3.3.2; R Core Team 2016) with packages “glmmADMB”, “multicomp”, and “MuMin”. The statistical significance level (α) was set at $p=0.05$. In generalized linear mixed models (GLMMs) except those on body size, we incorporated initial glochidia concentration during infection as a covariate to account for the potential effects of initial infection probability (Table 1). Log-likelihood tests were used to compare models whereas Tukey’s post-hoc multiple comparison tests were conducted when appropriate.

First, we compared the body sizes of fish used in different treatments. We ran Pearson’s correlation tests among three body-size measurements (i.e., fork length, standard length, and wet body mass) separately for fish collected on two occasions (i.e., infection and encystment), and found that wet mass was highly correlated with the two other body-size measurements on both occasions ($r > 0.95$, $p < 0.01$, in both cases).

Because of how closely related these measurements were, we used wet mass as a measure of body size. We developed a GLMMs with wet mass as a response variable, three main factors (i.e., strains of mussels and fish, and their interaction), and sampling occasion as a random factor (error distribution: Gaussian). We compared the model with (full model) and without the interaction term (1^{st} level reduced model). If the interaction term was insignificant, the 1^{st} level reduced model was compared with each of the models containing one of the main-factor variables (2^{nd} level reduced models).

Secondly, we examined the differences in initial glochidia infection among treatments. We developed a GLMM to examine if the sympatric pair showed the highest infection rates, with glochidia abundance attached to each gill as a response variable, three variables as main factors (strains of mussels and fish and their interactions), fish identity as a random factor, and gill surface area as an offset term (error distribution: negative binomial). We were interested in the interaction term, the significant effect of which would partially support hypotheses that the importance of the specific strain of mussel differed across cases using different fish strains. Model testing was done as with body size comparisons.

Thirdly, we examined the initial infection rates in relation to deformed gills among fish strains and their controlling factors. We developed a GLMM to examine if the deformed gill area affected the initial glochidia infection with glochidia abundance attached to each gill as a response variable, the proportion of deformed area in each gill as a main factor, fish identity nested within fish strain as a random factor, and gill surface area as an offset term (error distribution: negative binomial). We then developed

294 a GLMM to determine if the rate of gill deformity was associated with the size
295 differences in host fish, with the proportion of deformed area in each gill as a response
296 variable and wet body weight as an explanatory variable. We furthermore developed a
297 GLMM to examine if the glochidia density was affected by the condition of gill
298 filaments at the location of the attachment, with glochidia abundance of each gill in
299 each condition category (normal or deformed) as a response variable, the condition of
300 the attachment location as a main factor, fish identity nested within fish strain and
301 mussel strain as a random factor, and gill areas in each category as an offset term (error
302 distribution: negative binomial). In these three analyses, we compared models with
303 reduced models without the effects of main factors.
304 Lastly, we examined the cyst density and encystment rates of initially infected
305 glochidia to cysts in relation to fish and mussel strains. We developed a GLMM to test if
306 the sympatric pair showed the highest cyst density with cyst abundance in each gill as a
307 response variable, three variables as main factors (i.e., strains of mussels and fish and
308 their interactions), fish identity as a random factor, and gill surface area as an offset
309 term (error distribution: negative binomial). Gill area for each fish used in this model
310 was estimated from weight-area relationships obtained from the initial glochidia
311 measurement ($r^2>0.63$, $p<0.001$). We also developed a GLMM with the encystment rate
312 as a response variable, three variables as main factors (i.e., strains of mussels and fish
313 and their interactions), fish identity as a random factor (error distribution: Gamma
314 binomial). Encystment rate was obtained by dividing the cysts' abundance by the mean
315 of glochidia abundance for individuals obtained from each set of nine treatment

316 combinations. Model testing was done as with comparisons of initial infection rates in
317 relation to deformed gills.
318
319
320 **Results**
321 Glochidia infection density differed among groups as indicated by a significant effect of
322 interaction between strains (Table 2a). Multiple comparisons among groups showed
323 highest density for the treatments with Chitose mussels for Abira fish, Shakotan mussels
324 for Chitose fish, and indistinguishable levels across all the mussels infected with
325 Shakotan fish (Fig. 1). Host fish size used in the experiment differed among groups as
326 indicated by the significant effect of interactions between strains of fish and mussels
327 (Table 2b). The size of fish sub-sets from each river were similar to each other within
328 each mussel strain group except in one instance. The fish subset that the Abira River
329 provided to mussels from the Chitose River was larger than that provided to the Abira
330 fish. When fish size is compared among nine combination groups, Shakotan fish
331 provided to Abira and Chitose rivers were significantly smaller than Abira fish provided
332 to mussels from the Chitose and Shakotan Rivers (Fig. S5).
333 Glochidia density was significantly related to the proportion of deformed gill
334 area (Table 3a). With an increasing proportion of deformed area, glochidia infection
335 density decreased (Fig. 2a). The proportion of deformed gill area was higher for
336 Shakotan fish compared to other fish, and this tendency was explained by a negative
337 relationship between wet mass and the proportion of deformed areas on gills (Table 3b;

Figs. S5, 2b). The differences of infection density between the deformed and the normal parts of gills varied across fish strains (Table 4a). Glochidia density was significantly higher in normal gills compared to those in deformed gills for each fish strain, with the density in normal gills being lowest for Shakotan fish, highest for Abira fish, and intermediate for Chitose fish (Fig. 3).

Cyst density did not differ among any combination groups (Table 4b; Fig 4a). Encystment rate was affected only by fish strain; fish from the Shakotan river displayed a significantly higher rate compared to the fish from other rivers (Table 4c; Fig 4b). By examining field-collected fish in a similar way, we could not find any evidence of gill deformation in any of the rivers. All fish collected in the field had size parameters within the ranges of values recorded for the samples for initial infection in the experiment.

Discussion

A well-controlled laboratory experiment did not support our hypothesis that sympatric pairs of mussels (*M. laevis*) and host fish (*O. masou masou*) interact with higher success rates at both stages. An unexpected heterogeneity in pathological conditions in gills explained a greater amount of variance in these processes. This heterogeneity reduced the initial infection rate and increased the encystment rates of initially attached glochidia to cysts. Considerable efforts have been made to elucidate the underlying reproductive ecology of host-affiliate relationships between unionid freshwater mussels and host fish. Our results shed light on one of the least studied factors, providing an

empirical underpinning to the importance of pathologically diversified host conditions in the reproductive processes of unionid mussels.

Although conclusive causes remain unclear, a history of sympatric occurrence or contact possibly in relation to genetic background resulted in variability within the infection rate and the success of encystment and metamorphosis. Taeubert et al. (2010) reported greater success with a higher cyst abundance in a case where *M. margaritifera* was provided with fish in a sympatric habitat whereas Österling and Larsen (2013) demonstrated that sympatric pairs did not result in higher rates compared to other combinations. We predicted that fish and mussel pairs from the same collection localities would exhibit the highest rates of infection and encystment success as proposed in a theory of local adaptation (Kawecki & Ebert, 2004), because mussels might be selected to develop traits adapted to increase their reproductive rates with locally available fish. Akiyama (2007) conducted a cross-infection experiment using combinations of fish and mussels from two rivers (Chitose and Abira River) and showed results suggestive of sympatric pairs being the most successful. The weakness of their study was the small number of replicates and the lack of statistical tests on this aspect. Our study using individuals within a geographical range of both species in a fully factorial design refuted the notion that the population-level adaptations in these host-affiliate relationships is predictable based on the sympatricity of these species. Even for data from two rivers (Abira River and Chitose River) in which the potential effects of artificial fish stocking were not concerned and the prevalence of gill deformity was low, there was no evidence that sympatric pairs were more successful. *Oncorhynchus masou*

382 *masou* is known to form geographically distinct population structures (Kitanishi et al.,
383 2018) because of their strong tendency to return to their natal river (Kitanishi et al.,
384 2007). There is currently no such quantitative data on the genetic structure of pearl
385 mussels. From a theoretical viewpoint of local adaptation in parasites-host relationships,
386 a much longer life span of mussels (several decades up to >100 years) relative to that of
387 host fish (<several years) might prefer the selection of host adaptation to parasite
388 adaptation (Taeubert & Geist, 2017). A sufficiently long time might have not yet elapsed
389 for them to develop adaptive population-level infectivity or immunity. Instead, infection
390 of fish by glochidia or the failure of glochidia encystment may not play strong roles in
391 determining the fitness of both species.
392 The effects of gill deformities on the glochidia infection processes can be
393 attributed to the traits of glochidia that are used in their attachments to host fish. *M.*
394 *laevis*, like other species such as *M. margaritifera*, belonging to the same genus; they
395 can only parasitize in high abundance on the gills of host fish (Kondo, 2008; Ziuganov
396 et al., 1994). This is probably related to their small size and the absence of teeth-like
397 attachment organs (Akiyama, 2007; Kondo, 2008). Furthermore, Bauer (1987) noted
398 that *Margaritifera* glochidia attach to relatively soft-surfaced tissue in gills (see also
399 Araujo & Ramos, 2000), and that an increased hardening and thickness level of gills
400 with body growth may hamper the attachment of glochidia. Our results clearly showed
401 that the infection rate was substantially reduced in the gill surface with a symptom of
402 inflation and partial conglutination of gill filaments and lamella regardless of fish strain.
403 Although we did not examine the cause of it, the symptom suggests microbial infections

404 from *Flavobacterium* spp. or infections from small-sized ciliated protozoans (e.g.,
405 *Chilodonella piscicola, Ichthyobodo necator*) (Deng et al., 2015; Goldes et al., 1988;
406 Kimura, Wakabayashi, & Kudo, 1978). Fish observed in the field did not show such
407 deformities in their gills and the deformity was already present in the measurement of
408 initial glochidia infection immediately after the treatment even in the area without
409 glochidia attachment. Therefore, it is likely that the symptom occurred during the
410 incubation (acclimation) period in the rearing tank. A remarkably high prevalence of
411 deformed gills was seen in Shakotan fish, despite the fact that the three subsets were
412 reared separately and provided with a common water source across all the tanks. This
413 points to the possibility that this fish was predisposed to be susceptible. This fish had
414 the smallest observed body size for the strain, which might contribute to its
415 susceptibility. A small-sized body might be generally susceptible to the infection and
416 associated with low post-natal immunity (Johnson, Flynn, & Amend, 1982), and the
417 infection might have proliferated quickly among individuals at a much faster rate.
418 When assessed at the stage of encystment, the variations in infection rate
419 disappeared, resulting in similar cyst abundance across all the groups. This was
420 explained by an increased level of the encystment rate of initially attached glochidia for
421 fish from the Shakotan River. In the process of initial attachment to and encystment on a
422 compatible host, immune responses gradually reduce the numbers of glochidia over
423 time (Österling & Larsen, 2013). Therefore, it is likely that pathogenically affected
424 individuals did not exhibit strong immunity. Among the known factors that could affect
425 host immunity to glochidia infection are temperature (Roberts & Barnhart, 1999;

Taeubert et al., 2014), infection history (Rogers & Dimock, 2003), and body condition (Douda et al., 2018). Temperature and history are the unlikely reasons because water tanks were provided with a common water source, and fish classified as 0+ were collected before the known period of mussel reproduction and thus without previous encounters. The most probable cause was the condition of host fish. First, fish immunity might have been reduced because of the gill conditions. Douda et al. (2018) showed that host fish treated under high-stress environments were more susceptible to glochidia infection, which is consistent with our explanations. Second, small body-size fish were characterized with a lower level of immunity and thus retained more cysts because of the possible presence of size-dependent post-natal immunity (Johnson et al., 1982). In the current experimental setting, both hypotheses needed to be retained.

In conclusion, we demonstrated that affiliate-host relationships between *M. laevis* and *O. masou masou* at the two stages in the reproduction process were not affected by population-level characteristics developed through their sympatric occurrences, and thus showed no evidence of local adaptation. Overall, this study provided one of the strongest empirical supports for the first evolutionary adaptation pattern proposed by Taeubert and Geist (2017) for the freshwater pearl mussel and its host. The unexpected and new knowledge from this study is that a gill deformity can affect initial infection rates possibly via complex immunity-related responses. From a perspective of artificial culturing programs, our results suggest that the use of different sources of host fish would not substantially affect the variability of reproductive success for *M. laevis*. However, the host-dependent reproductive process of *M. laevis* extends

until the excystment period. Further reduction of some cysts may continue until the completion of metamorphosis and excystment (Österling & Larsen, 2013). Our measurement timing for encystment was before the known timing for *M. laevis* (approximately 40-50 days in comparable temperature; Akiyama, 2007; Kondo, 2008). Thus, further studies are needed to determine how the observed similar encystment abundance among groups will be reflected in population parameters such as growth and survival of juveniles. Encystment conditions may have lingering effects on juveniles (Marwaha et al., 2017). Infected fish may also show diverse patterns after glochidia infection (Ooue et al., 2017; Terui et al., 2017), and the presence of deformities may lead to different outcomes. If the increased rate of the encystment and cyst abundance was purely associated with body size-dependent immunity, the highest cyst abundance might be obtained by infecting smaller fish from the Shakotan River in a pathogenic-deformity-free environment.

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Table 1. The concentration (number of individuals/L) of glochidia in water used for infection in each combination of experimental treatments. Concentration was estimated based on the averages of 10 replicated counts of glochidia in 250-ml sample water from each tank

Mussel strain	Fish strain		
	Abira	Chitose	Shakotan
Abira	46500	40500	45000
Chitose	37500	27000	43500
Shakotan	33000	31500	31500

Table 2. Results of GLMMs testing the effects of strains of fish and mussel and their interactions on initial glochidia abundance in each gill of *O. masou* (a) and testing the differences in host fish size among combinations of experimental treatments (b). Full models and reduced models were each compared using a log-likelihood test. Fish (F) and Mussel (M) denote fish strain and mussel strain, respectively

(a)	logLik	AICc	p-value
Full model			
Fish (F), mussel (M), F×M	-1742.76	3514.7	**<0.001**
Reduced model			
F, M	-1753.34	3527.3	

(b)	logLik	AICc	p-value
Full model			
Fish (F), mussel (M), F×M	-123.749	272.9	**<0.05**
Reduced model			
F, M	-130.105	275.6	

Table 3. Results of GLMMs testing the effects of areal proportion of deformed gill surface area on initial glochidia abundance in each gill of *O. masou* (a) and the effects of wet mass of host *O. masou* on the areal proportion of deformed gill surface area of *O. masou* (b). P-values were obtained from Z-tests; SE denotes standard errors. Initial glochidia refers to the average concentration of glochidia water used in infection treatments

(a)	coefficients	SE	p-value
Proportion of deformed area	-2.8×10^{-2}	3.2×10^{-3}	**<0.001**
Initial glochidia	-1.6×10^{-5}	6.3×10^{-6}	**<0.05**

(b)	coefficients	SE	p-value
Wet mass	-3.2×10^{-2}	7.2×10^{-3}	**<0.001**
Initial glochidia	-2.5×10^{-7}	7.8×10^{-7}	0.75

Table 4. Results of GLMMs testing the effects of strains of fish and the condition of infected area and their interactions on initial glochidia abundance in each gill of *O. masou* (a), testing the effects of strains of fish and mussel and their interactions on encysted glochidia (cyst) abundance in each gill of *O. masou* (b), and testing the effects of strains of fish and mussel and their interactions on the rate of encystment of initially attached glochidia in each gill of *O. masou* (c). Full models and reduced models were each compared using a log-likelihood test; when Full model was insignificant, 1st reduced models were compared to 2nd reduced models and sequentially to the null models. Fish (F) and Gill (G) denote fish strain and gill condition of glochidia attachment, respectively. Superscripts of p-values indicate the variables removed from the model to test with those from reduced models by one level

(a)	logLik	AICc	p-value
Full model			
Fish (F), Gill (G), F×G	-1951.01	3922.4	<**0.001**
Reduced model			
F, G	-130.105	4004	

(b)	logLik	AICc	p-value
Full model			
Fish (F), Mussel (M), F×M	-1249.96	2529.1	0.29
1st Reduced model			
F, M	-1252.41	2525.5	0.17 ^{F}, 0.21^{M}
2nd Reduced model			
F	-1254.2	2524.8	0.22
M	-1253.96	2524.3	0.17
Null model			
	-1255.71	2523.7	

(c)	logLik	AICc	p-value
Full model			
Fish (F), Mussel (M), F×M	1618.08	-3211.3	0.67
1st Reduced model			
F, M	1616.92	-3217.4	<**0.01** ^{F}, 0.79 ^{M}
2nd Reduced model			
F	1616.68	-3221.1	<**0.01**
M	1612.34	-3212.4	0.81
Null model			
	1612.13	-3216.1	

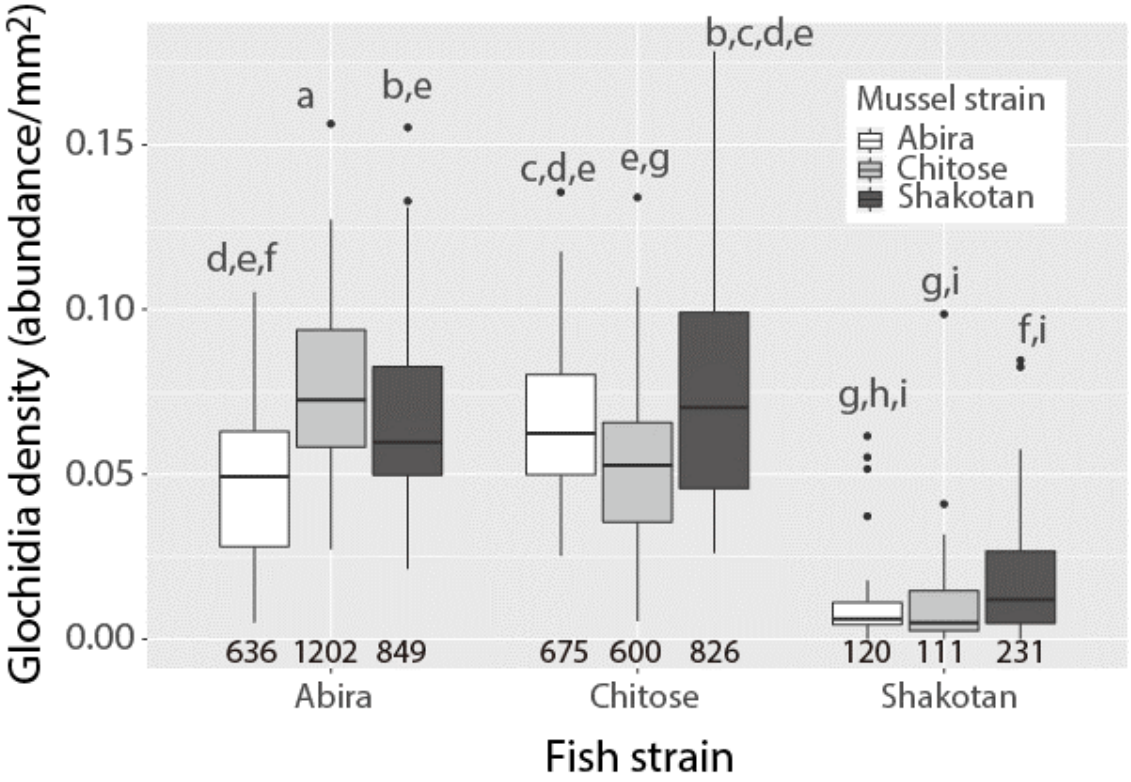
Figure legends

Figure 1: Glochidia density on each gill compared across all the strain combinations at an infection stage (within one hour of infection). Results of multiple comparisons of each were shown; those accompanied by the same alphabetical letters were considered statistically the same. Boxplot legend: top (bottom) edges of box are 75th (25th) percentiles; center line in box is median; the upper (lower) whisker extends from the box edge to the largest (smallest) value no further than 1.5×inter-quartile ranges of the edge; data beyond the end of the whiskers are outliers and are plotted individually. Numbers below boxes denote mean abundances of glochidia per fish for each treatment

Figure 2: Glochidia density on each gill, which was the abundance of glochidia divided by total surface area of each gill, in relation to the proportion of deformed gill surface area in each fish (a) and the proportion of deformed gill surface area in each fish in relation to wet body size of fish (b). Dotted lines represent statistically significant model regression lines

Figure 3: Glochidia density on each gill, which was the abundance of glochidia divided by total surface area of each gill, in relation to the condition of the attached gill surface. Box-plot legends as in Figure 1. Results of multiple comparisons among groups were shown; those accompanied by the same alphabetical letters were considered statistically the same

Figure 4: Encysted glochidia (cyst) density on each gill, which was the abundance of cysts divided by total surface area of each gill (a) and encystment rate obtained from initially infected glochidia to cyst (b). Box-plot legends as in Figure 1. Results of multiple comparisons among fish strain groups were shown; those accompanied by the same alphabetical letters were considered statistically the same. Numbers below boxes denote mean abundances of cyst per fish for each treatment



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780 **Figure 1**

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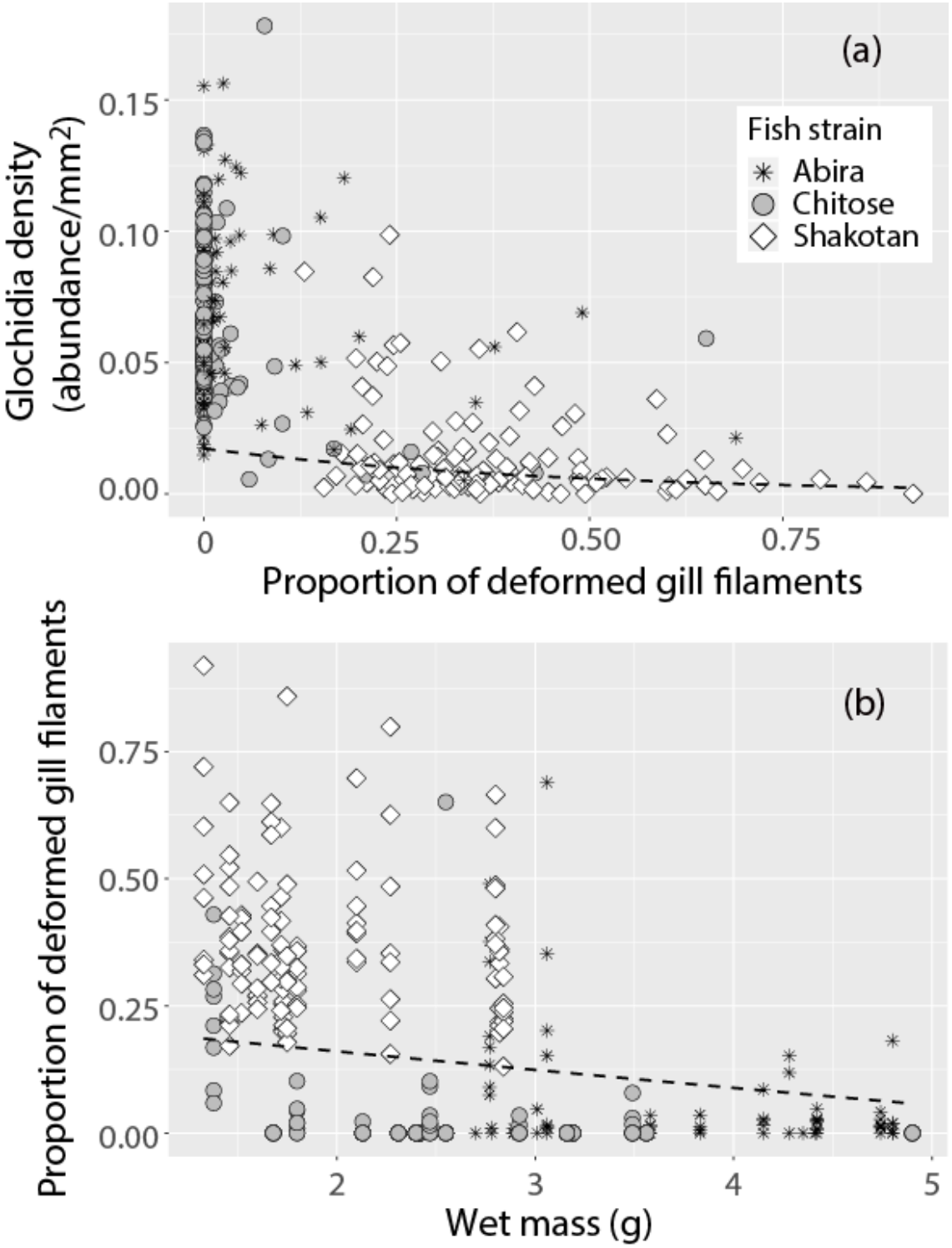

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784 **Figure 2**

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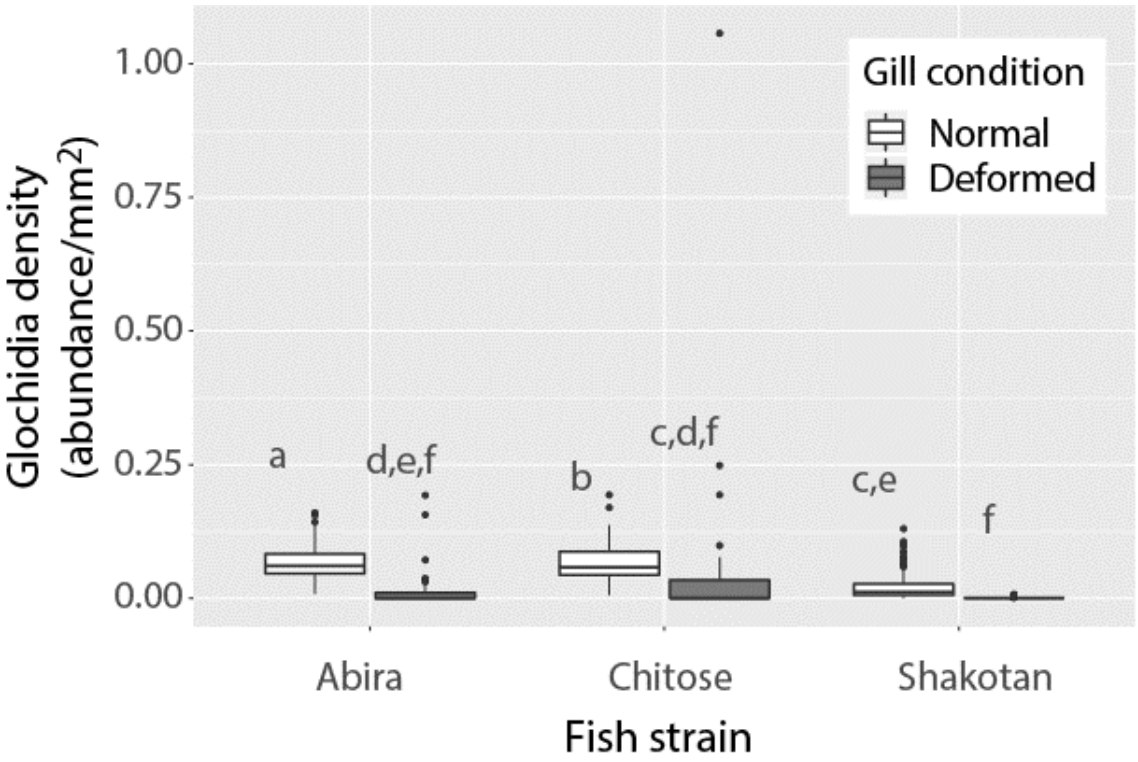

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788 **Figure 3**

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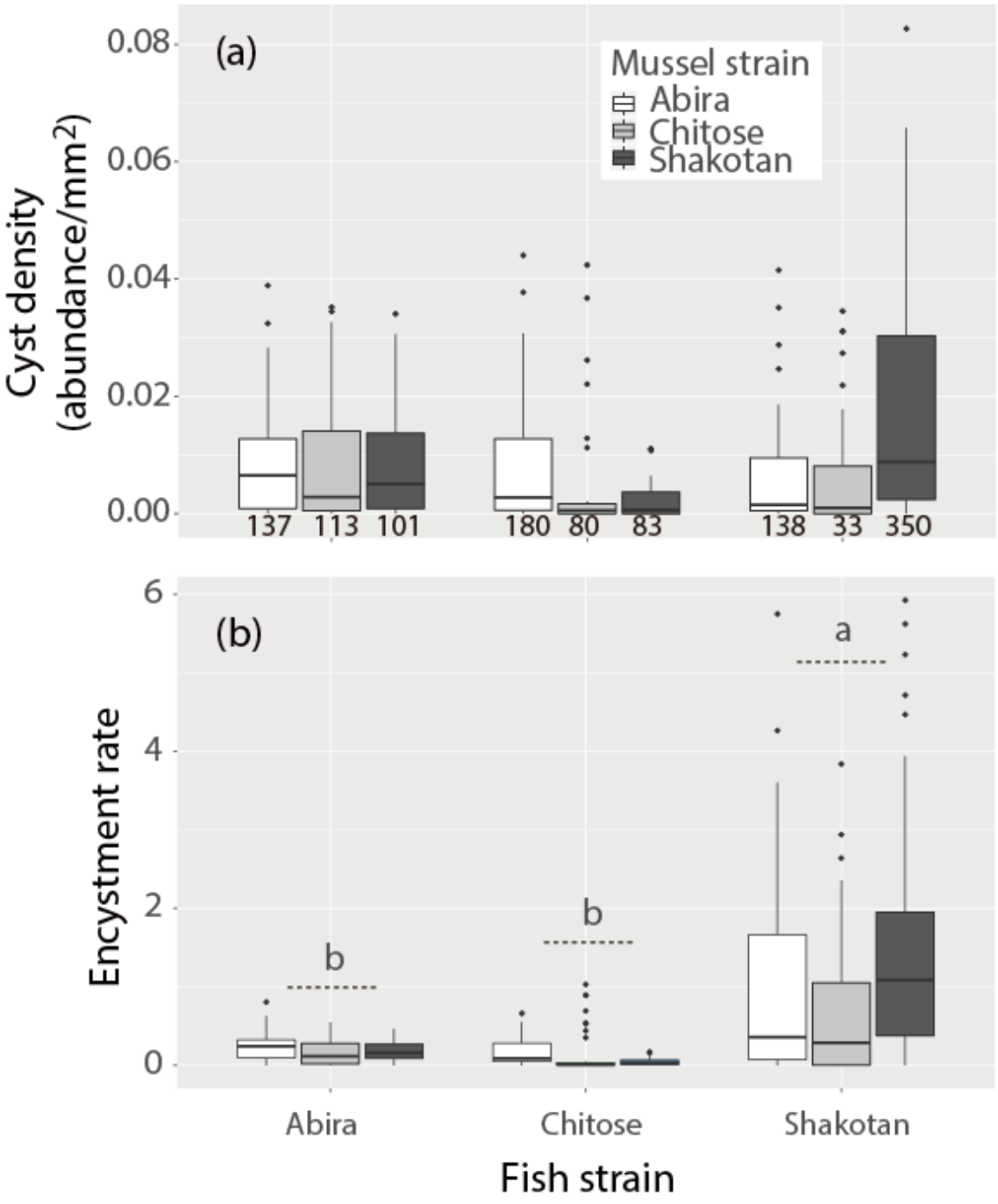

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792 **Figure 4**

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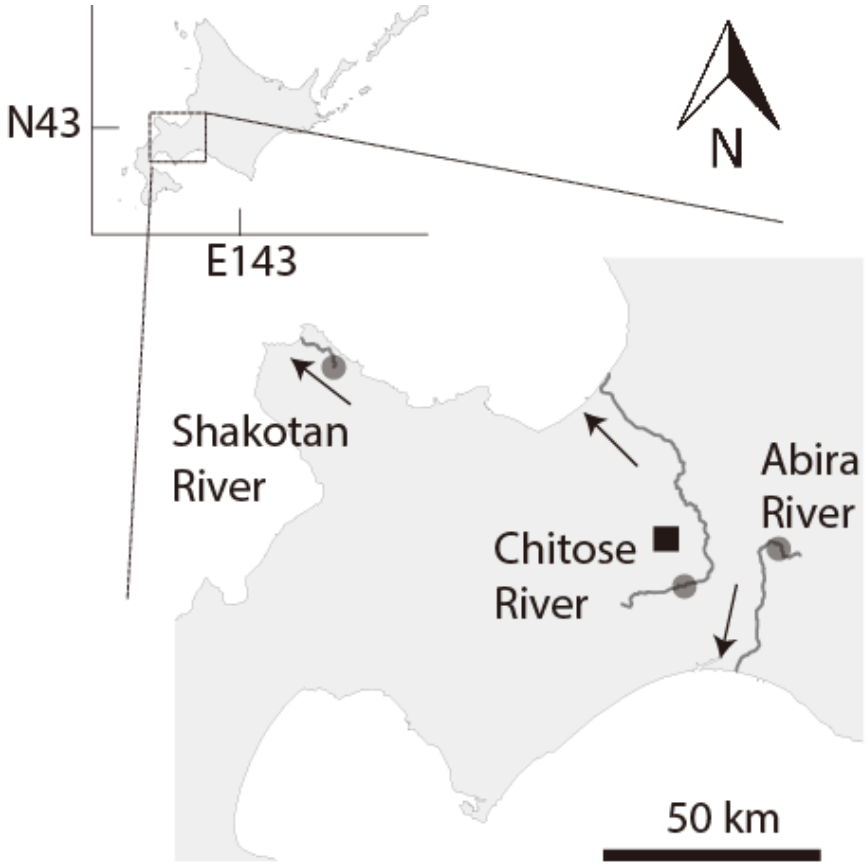
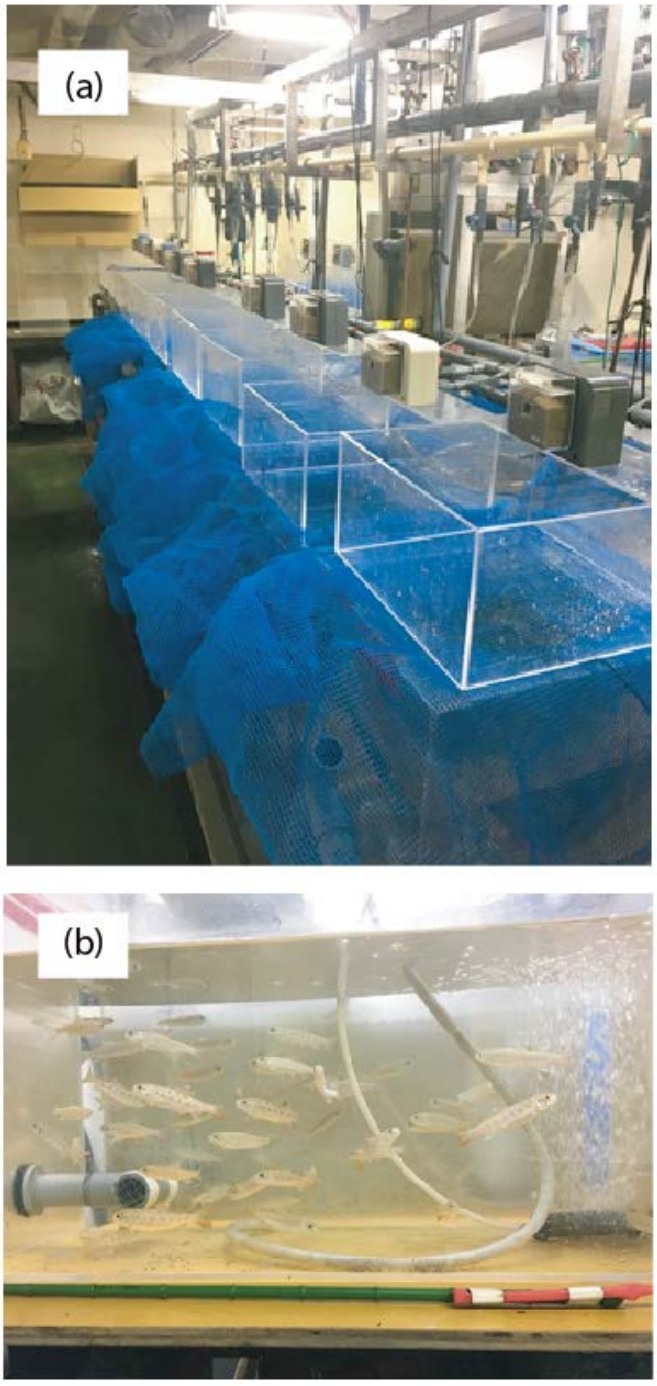


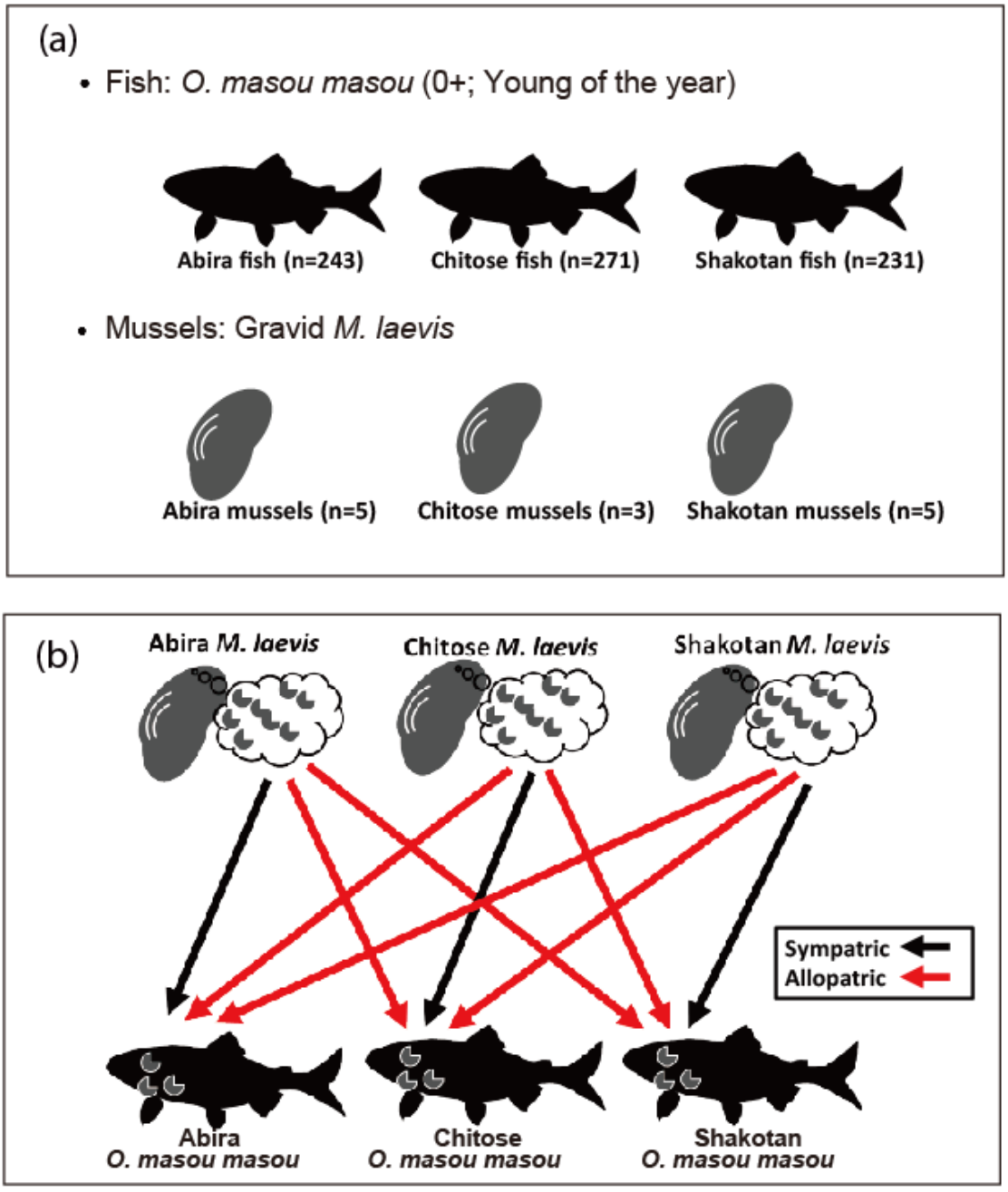
Location of study area, and three study rivers in Hokkaido, Japan. A filled square denotes the experimental facility where infection experiments were conducted whereas gray circle represent locations where fish and mussels were collected

Figure S1



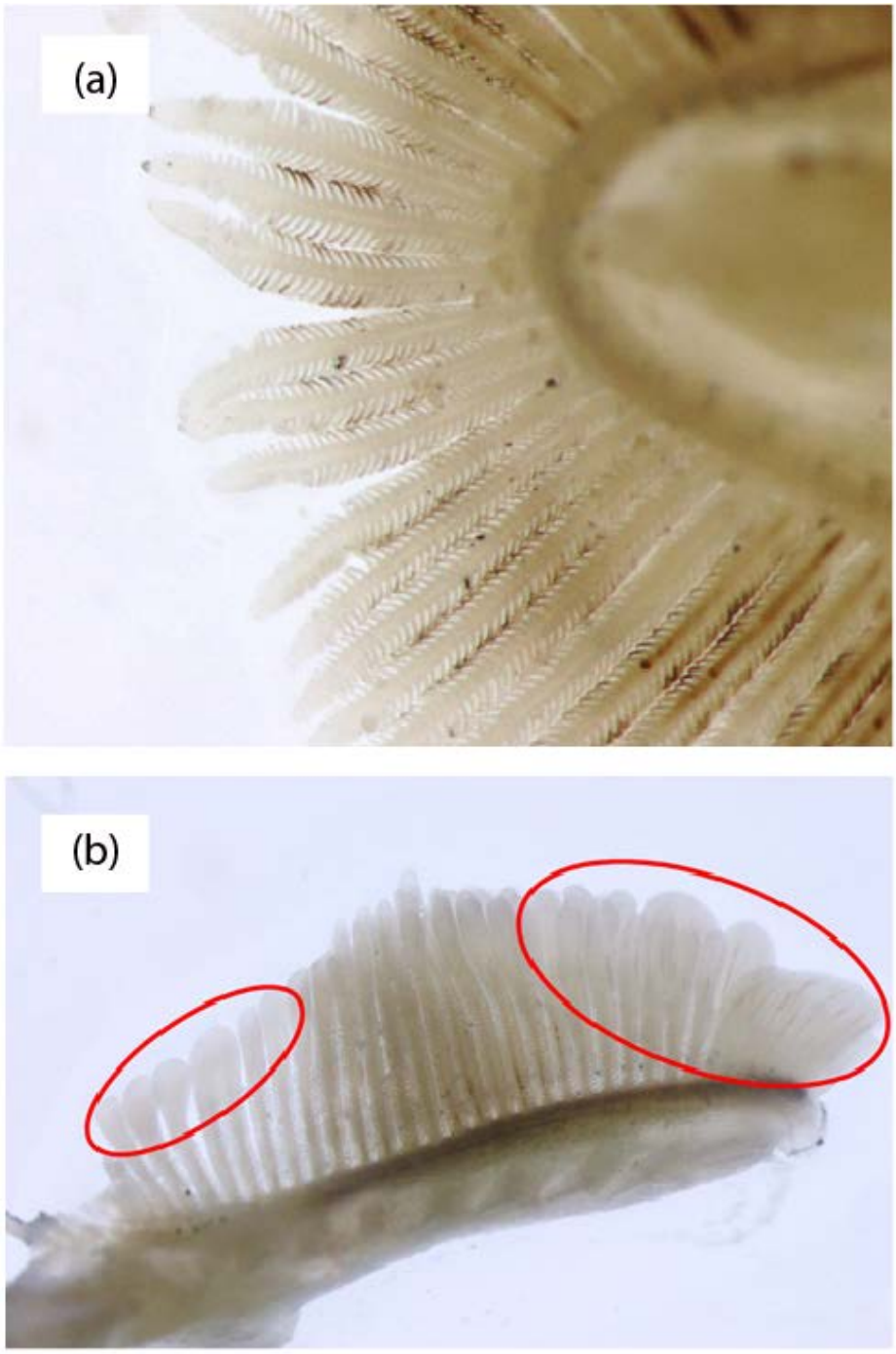
Experimental tanks equipped with automatic feeders (A) and an experimental tank during infection experiment (B)

Figure S2



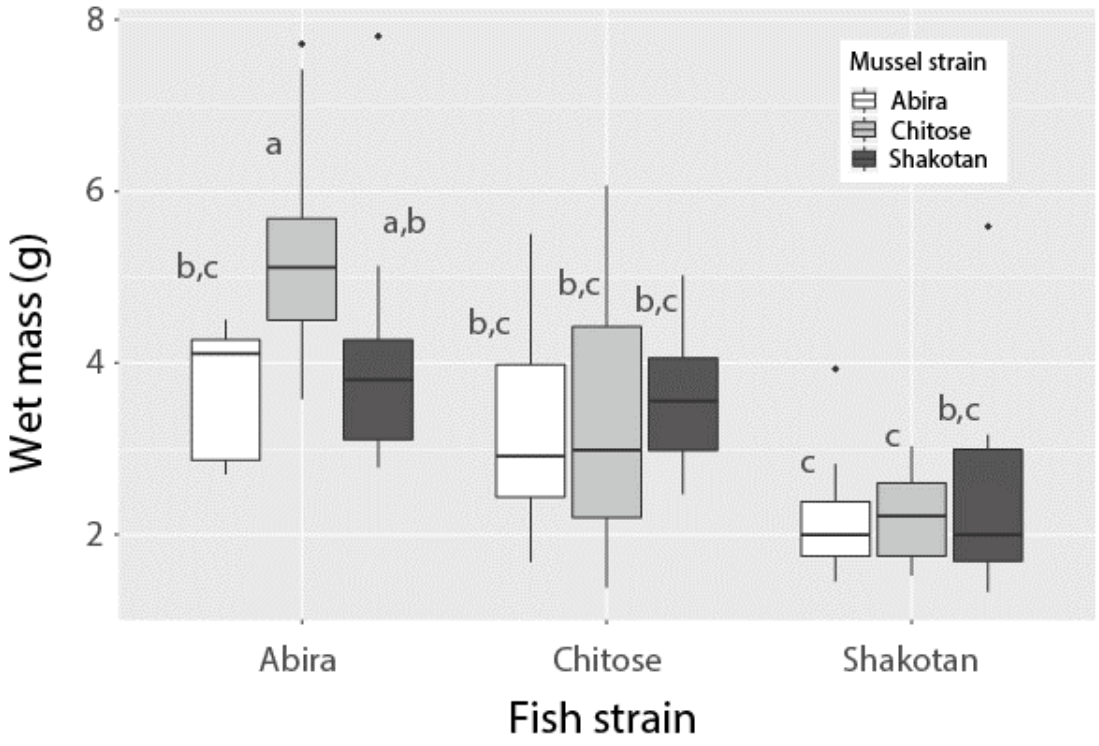
A schematic diagram showing materials used in the experiment (A) and nine combination treatments (tanks), which consisted of three strains of mussels × three strains of fish (B). In (B), 61 fish were used in each treatment

Figure S3



Photos showing normally developed gill lamellas and filaments (A) and inflated and partly conglutinated deformed gill lamellas and filaments (B)

Figure S4



Wet mass (g) of host fish used in each treatment groups. Results of multiple comparisons among groups were shown; those accompanied by the same alphabetical letters were considered statistically the same

Figure S5