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Population status and processes of recruitment failure of endangered freshwater pearl mussels (*Margaritifera togakushiensis*) in eastern Hokkaido, northern Japan

（北海道東部における絶滅危惧淡水二枚貝
コガタカワシンジュガイの個体群の現状と再生産の停止機構）

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Abstract

Freshwater mussel (order Unionida) is one of the groups of freshwater molluscs having complex life histories that require temporary parasitization on host fish. They have important ecological functions in freshwater ecosystems but became one of the most imperiled organisms worldwide. The identification and understanding of the causes and the processes of population declines or species extinction of freshwater mussels are imperative for effective conservation measures. Recent studies have shown that one of the major indications for the decline of populations of the freshwater mussels is recruitment failure, i.e. low levels or no signs of recent joining of juvenile mussels. These populations will become extinct in the near future if recruitment will not restart. To prevent the extinction of freshwater mussel populations, the process of recruitment failure of freshwater mussels should be urgently determined. A series of observational and experimental studies determined the population status and demonstrated the processes of recruitment failure of long-lived endangered freshwater pearl mussel *Margaritifera togakushiensis* in the rivers of eastern Hokkaido, northern Japan.

The study first investigated the co-occurrence distributions of *M. togakushiensis* and *M. laevis* (Chapter 2). *Margaritifera laevis* is closely related and has very similar appearances with *M. togakushiensis* despite having ecological differences in host fish species and reproductive seasons. The two species were identified by gel electrophoresis banding patterns of 16S rRNA polymerase chain reaction products. The results showed that *M. togakushiensis* was likely to co-occur with *M. laevis* at river reach scale (within 50–100 m). In addition, co-occurrence distributions of two pearl mussel species and populations of *M. togakushiensis* relatively well confirmed in eastern Hokkaido (Chapter 2). In response to the high possibilities of co-occurrence distributions between two pearl mussel species, I developed simple non-lethal identification criteria of two pearl mussel species based on linear discriminant functions (LDFs) that were established considering intraspecific regional morphological differences from east and west regions at Hokkaido (Chapter 3). The two species were also identified using DNA-analysis. Regardless of region, the maximum length of *M. laevis* exceeded 100 mm, whereas all *M. togakushiensis* were < 100 mm in length. The LDFs revealed

that the morphologies of the two species were clearly distinguished by the relationship between height and length. Identification accuracies of the established LDFs were high with 85–96% (mean: 92%) accuracy in the east and 67–96% (mean: 80%) in the west. Using these identification criteria, I investigated the status of 24 populations of *M. togakushiensis* in eastern Hokkaido (Chapter 4). A total of seven rivers were estimated as "functional" with more than 20% of ≤ 20-year-old juveniles contained whereas recruitment failure of *M. togakushiensis* was confirmed in four rivers where no juveniles were found. At maximum > 70 years remained until the extinction of those populations based on estimates deriving from age distributions. The estimated periods of recruitment cessation of non-recruiting populations well coincided with the timing of the development of the vast agricultural lands in the study region. Finally, I demonstrated the processes of recruitment failure of *M. togakushiensis* populations in eastern Hokkaido focusing on the major multiple reproductive life-history stages (Chapter 5). The reduced survival rate of experimentally provided juvenile abundances was associated with the amount of fine suspended sediment, the level of dissolved nutrients, and their interaction. Although there were no clear relationships between suspended sediment levels and agricultural land and urban proportions in catchments, the negative impacts of catchment agriculture and urban area were inferred as a cause. Furthermore, the lack of the potential number of juvenile supplies at the parasitic stage can also cause recruitment failure interactively with the juvenile survival rate. These results suggest that the recruitment of *M. togakushiensis* limited by multiple life-history stage processes.

This study is the first case that empirically demonstrated the processes of recruitment failure of long-lived freshwater mussel species by testing the multiple reproductive life-history stages of *M. togakushiensis* comprehensively. In conclusion, the post-parasitic juvenile stage is the most sensitive to habitat deterioration among the life histories of *M. togakushiensis* and can become a serious bottleneck for their recruitment. This study would provide perspectives for future conservation measures of freshwater mussels worldwide because the results suggest that greater attention should be paid to the order of conservation measures. To conserve populations of *M. togakushiensis*, the watershed management that reduces the inflow of nutrients is likely to be an effective option for recovering the recruitment of *M. togakushiensis* in eastern Hokkaido. In addition,

determining the origins of excessive fine sediments and reducing them is required. Furthermore, habitat improvement of host fish like installation of large wood and artificial propagations of *M. togakushiensis* may be also effective to enhance the potential numbers of juvenile supplies and thus recover natural recruitment.

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1. Introduction

Freshwaters comprise only 0.01% of the water on Earth. Despite of relatively small spatial coverage, freshwater ecosystems are known as one of the most diverse systems on which about 9.5% of the described animal species on Earth (~125 000 species) including one-third of vertebrates depend (Balian et al. 2008). However, freshwater ecosystems have been rapidly degraded by human impacts in recent decades (Dudgeon et al. 2006). The World Wide Fund for Nature (WWF) Living Planet Index (LPI), which is a measure of the state of world's biological diversity based on population trends of vertebrates species from terrestrial, freshwater, and marine habitats (Collen et al. 2009), showed that the index fell to 83% from 1970 to 2018 (WWF 2018) indicating that freshwater ecosystems are considered to be the systems with the most serious decline in biodiversity compared with other ecosystems (e.g. terrestrial and marine ecosystems). Major threats to the freshwater species include climate change, over-exploitation, habitat degradation, habitat fragmentation, invasions of exotic species, and flow modification (Dudgeon et al. 2006). Moreover, recent studies are warning that the new threats are emerging such as microplastics, noise and light pollution, engineered nanomaterials, and infectious disease (Reid et al. 2018). The identification and understanding of the cause and the processes of population declines or species extinction are imperative for effective conservation measures.

Freshwater mussels (order Unionida) are widely distributed in lakes, ponds, and rivers worldwide except for Antarctica (Graf and Cummings 2007, Carter et al. 2011). More than 800 freshwater mussel species have been described in the world (Graf 2013), and 17 species and 1 subspecies have also been confirmed in Japan (Kondo 2008). Freshwater mussels are functionally important for the maintenance of healthy ecosystem function of freshwater ecosystems because they modify the physical characteristics of their habitats including bed-sediment movements and as well as alter nutrient cycling (Gutiérrez et al. 2003, Zimmerman and de Szalay 2007, Vaughn and Hoellein 2018). They also serve as ecological indicators in freshwater ecosystems. For example, they integrate relatively long-term information such as nitrogen inputs into watersheds in their tissues because of their filtration foraging behaviors and sedentary lifecycle (Atkinson et al. 2014). However, declines of mussel diversity and/or populations have been widely reported in recent decades, and freshwater mussels became one of the most imperiled organisms worldwide

(Ricciardi and Rasmussen 1999, Lydeard et al. 2004). Of 536 freshwater mussel species assessed by the IUCN, 32 species have gone extinct, 216 species were categorized as Near-threatened, Vulnerable, Endangered, or Critically Endangered, and 83 species were classified as Data Deficient (IUCN 2019). In North America, 70% of freshwater mussel species are now endangered, threatened, or of special concern as well as in Japan (Negishi et al. 2008, Haag and Williams 2014). Their vulnerability to anthropogenic disturbances derived from the following three reasons. First, their growth is slow and they need to take for a long time to maturity. If once disappear, their recovery would take a long time (Strayer et al. 2004). Second, freshwater mussels are unlikely to escape dramatic environmental changes due to their low mobility (Ferreira-Rodríguez et al. 2019). Third, freshwater mussels can be affected not only by the direct effects of environmental change but also by the indirect effects of host fish because they have complex life histories that require temporal parasitization on host fish during their larval phase ("glochidium larvae", Haag 2012; see the section "The life histories of *M. togakushiensis*").

Recent studies have shown that one of the major indications for the decline of populations of freshwater mussel is recruitment failure, i.e. low levels of juvenile mussels joining populations for sustaining them for a long time. Many mussel populations without recently born juveniles within 10–20 years have become increasingly common worldwide (Geist 2010, Österling et al. 2010, Lois et al. 2014, Simon et al. 2015, Ferreira-Rodríguez et al. 2019). These populations will become extinct in the near future if recruitment will not restart (Österling et al. 2008, Geist 2010, Ferreira-Rodríguez et al. 2019). The recruitment should be reestablished by the timing of disappearances of reproducing mussel individuals to prevent the local extinction of mussel populations. However, there are few cases that have succeeded in establishing the recruitment of freshwater mussel species. For example, Galbraith et al. (2018) reported that the reintroduction of American eels (*Anguilla rostrata*), which is a migratory host fish of *Elliptio complanata*, could reestablish recruitment of *E. complanata* in only one site between two target sites where American eels were absent due to fragmentation by past dam installation. One of the reasons for the difficulties of reestablishing recruitment is the lack of understanding of the processes of recruitment (Galbraith et al. 2018, Haag et al. 2019), which is largely associated with their complex life history. Therefore, the process of recruitment failure of freshwater mussels should be determined to prevent the

extinction of freshwater mussel populations.

The target organism of this thesis is freshwater pearl mussel, *Margaritifera togakushiensis* (family Margaritiferidae), and this species is distributed in Sakhalin, Russia, and Nagano, Iwate, and Aomori Prefecture in Honshu and Hokkaido, Japan (Kondo 2008). *Margaritifera togakushiensis* typically inhabits sandy to gravelly bottom in streams where the water temperature remains below 20-degree Celsius and White-spotted char, *Salvelinus leucomaenis leucomaenis* (family Salmonidae), occur as their host (Kondo 2008). *Margaritifera togakushiensis* has recently decreased due to the anthropogenic disturbances such as deforestation, construction of dams and wires, and river modification (e.g. straightening, canalization, and bank protection), thus this species has been listed as "endangered species" in the Red List of Japan (Ministry of the Environment of Japan 2018). However, the processes of declining *M. togakushiensis* populations have not been investigated yet.

It is possible that recruitment failure of *M. togakushiensis* is an undergoing phenomenon because closely related other *Margaritifera* species are being endangered for the same reason (Akiyama 2007, Geist 2010, Miura 2015). *Margaritifera* spp. including *M. togakushiensis* is one of the most long-lived groups among unionoids with more than 100–200 years maximum age (Degerman et al. 2009, Haag and Rypel 2011). Even after the cessation of reproduction, this species will survive for a long time because of its longevity. Without further recruitments, they would become extinct when all reproducing adults disappear. Thus, the current population status including recruitment (recruitment success/failure or the proportion of juveniles within a population) and the cause of declines of this species are needed to effectively conserve the species (Miura et al. 2019a).

The life histories of *M. togakushiensis*, and research gaps

Freshwater mussels including *M. togakushiensis* are hermaphrodite. Matured females inhale the sperm sphere released from matured males through inhalant siphons, and they fertilize and produce glochidium larvae in their gills in the reproductive season (March to May) (Fig. 1-1, Kondo 2008, Kobayashi and Kondo 2009). Matured glochidia are released to the water column through exhalant siphons of females and parasitize on the gills of *S. leucomaenis leucomaenis* for 40–50 days. The juveniles detach from the host tissues after metamorphosis and live in the sediments of the streambed. After several years to decades, they move to the stream-bed surface.

The recruitment of *M. togakushiensis* can be stopped or slowed when any life-history stages are impaired by human activities. Previous studies have investigated the cause of recruitment failure of freshwater mussels. Major reasons for recruitment failure are habitat destruction, fragmentation, and habitat degradation, including the decline or disappearances of host fish species (Österling et al. 2008, 2010, Strayer and Malcom 2012, Österling and Hogberg 2014, Galbraith et al. 2018). However, most previous studies have only focused on a part of their life histories (Buddensiek 1995, Gascho Landis and Stoeckel 2016, Haag et al. 2019). These studies haven't comprehensively tested the relationship between each life-history stage and environmental factors. In addition, previous studies have suggestively shown the juvenile stage is the most sensitive stage to the environmental change among their life histories (Österling et al. 2008, 2010, Strayer and Malcom 2012, Haag et al. 2019). However, little is known about the ecology of the juvenile stage of freshwater mussels. Thus, the processes of how any human impacts having a relationship with recruitment failure inhibit the recruitment of freshwater mussels have not been fully understood yet (Haag et al. 2019). Therefore, the following studies are needed for understanding the processes of recruitment failure of *M. togakushiensis*.

1. The study that determines current population status, in particular, recruitment (i.e. the proportion of juveniles, which is an indicator of recruitment efficiency of freshwater mussels, Strayer and Malcom 2012) and age structure to clarify which population has no or low proportion of juveniles.
2. The study that determines key life-history stage limiting recruitment comprehensively by testing the relationship between the performances at major reproductive life-history stages including juvenile stage, and recruitment.

Study aim and study design

In this thesis, a series of studies were conducted to demonstrate the processes of recruitment failure of *M. togakushiensis*. Most known distributions of this species in Japan are in Hokkaido region (Machida and Akiyama 2013, Akiyama et al. 2014, Sakai et al. 2017, Miura et al. 2018b, 2019a). Thus, I targeted this region. One of the important challenges for the achievement of

the aim is the difficulty of species identification between two pearl mussel species: *Margaritifera laevis* and *M. togakushiensis*. *Margaritifera laevis* is closely related to *M. togakushiensis* and it is also distributed in Japan having a range widely overlapping with *M. togakushiensis* (Kondo 2008, Sakai et al. 2017). The misidentification of these two species could lead to different consequences for the planning of the conservation measures because of the major differences in their reproductive timing and host salmonid fish. *Margaritifera laevis* reproduces in summer (June to Autumn) (Kurihara and Goto 2011, Terui et al. 2015), with *Oncorhynchus masou masou* (*O. masou ishikawae* in western Japan) as their hosts (Kondo 2008). The difficulty of species identification of two pearl mussel species has partially inhibited progressing the ecological studies including the distribution of the species. The ecology of *M. laevis* has been relatively well studied in Japan because several single or dominated populations of *M. laevis* are known (Akiyama 2007, Akiyama and Iwakuma 2010, Terui et al. 2014b, 2014a, 2015) while *M. togakushiensis* was only recently described as a distinct species on the basis of the differences of gene, morphology, and host-use (Kurihara et al. 2005, Kondo and Kobayashi 2005). Thus, I relied on DNA analyses throughout the thesis to maintain a high level of accuracy in the identification of the target species.

The thesis consists of six chapters, in which four chapters, Chapters 2 to 5, examined different aspects related to research objectives were provided and followed by the last Chapter 6 on overall conclusions. Specifically, in Chapter 2, I firstly examined co-occurrence distributions of two freshwater pearl mussels (*M. laevis* and *M. togakushiensis*) in a river reach in rivers in Hokkaido, northern Japan. This was an important step for clarifying the potential region of co-occurrences of two pearl mussel species. If both species widely co-occur in the same river reach, there was a need for establishing nonlethal species identification criteria in the next step. In Chapter 3, I developed simple nonlethal identification criteria for two freshwater pearl mussels in Hokkaido based on the results of Chapter 2. In Chapter 4, I determined the detailed population status of *M. togakushiensis* including age distributions and the recruitment using identification criteria developed in Chapter 3. In Chapter 5, I identified the processes of recruitment failure of *M. togakushiensis* by intensive field observations and experiments. In Chapter 6, I finally discussed what is important for the conservation of freshwater mussel species worldwide and *M. togakushiensis* in the study region and drew overall conclusions.

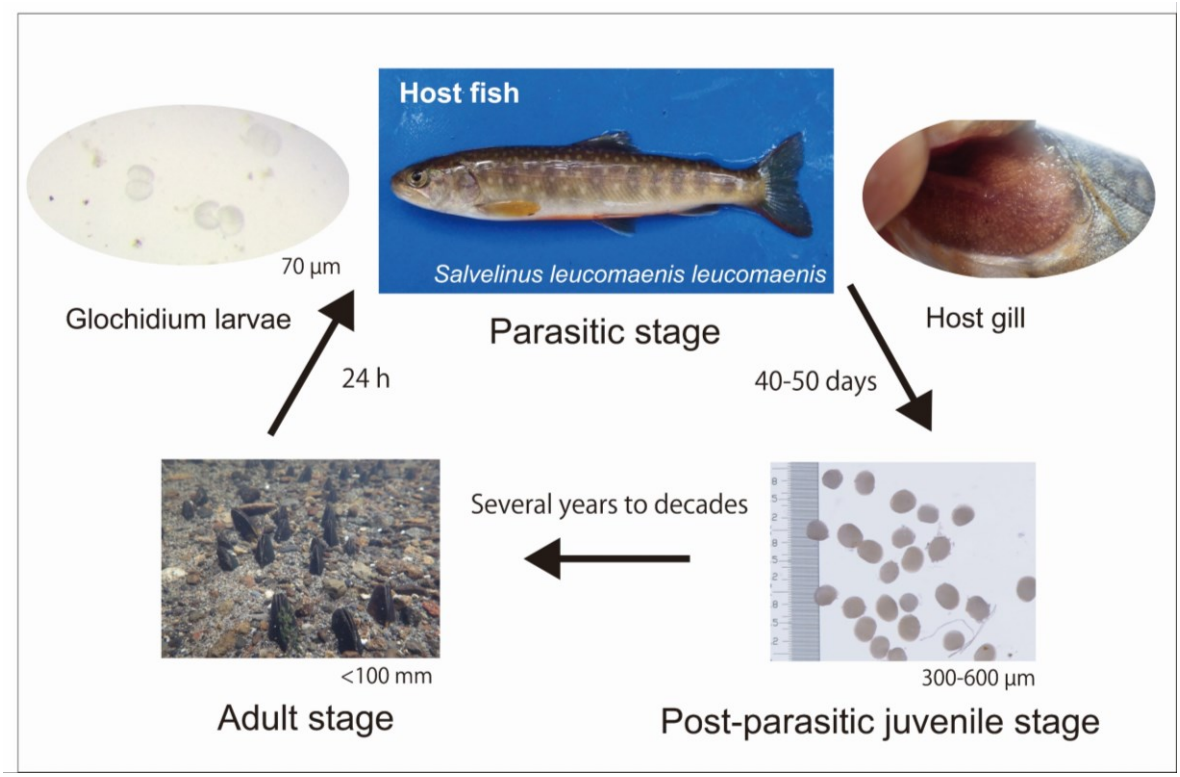


Fig. 1-1 Life histories of *M. togakushiensis*.

2. The co-occurrence of two endangered freshwater pearl mussels (*Margaritifera laevis* & *M. togakushiensis*) in rivers in Hokkaido, northern Japan

2-1. Introduction

Two or more species having different ecological characteristics each other may need different habitats although they are closely related and occur sympatrically in rivers and ponds (e.g. Onikura and Nakajima 2012). In such situations, the managers need to take proper actions that fulfill the habitat requirements of target species. Also, researchers can address various questions by considering species-specific habitat requirements. The assessment of species distribution including sympatric co-occurrence of multiple species is helpful because it could provide knowledge that allows managers and researchers to know in priori whether this needs to be considered.

Two freshwater pearl mussel species, *Margaritifera laevis* and *M. togakushiensis*, are related species to each other (Kurihara et al. 2005). However, they have ecological differences such as host salmonid fish species and reproductive seasons (Kondo 2008, Kurihara and Goto 2011, Terui et al. 2015). If the two species co-occur, it is necessary to take into account their differences in ecological characteristics when conducting the investigation and conservation of the two species. Furthermore, these species' appearance is similar to each other, further underscoring the usefulness of prior knowledge about probable co-occurrence of them to prevent flawed conclusions from research and implications to management.

Previous studies have shown that the two pearl mussel species co-occur within the same river segments (i.e. spatial scales of several to several ten kilometers) (Takeuchi et al. 2007, Sakai et al. 2017). However, freshwater mussels including freshwater pearl mussels are sedentary, and their dispersal distance is typically low with several meters in a year during their adult stage (e.g. Balfour and Smock 1995). Kurihara and Goto (2011) showed the annual dispersal distance of adult individuals of *Margaritifera* spp. in Japan is also within several ten meters (but this result includes dislodgement of individuals). In addition, dispersal distances of the host fish of *M. laevis* (*Oncorhynchus masou masou*) during their parasitic periods were mostly within 100 m up- and down-stream distances (Terui et al. 2014a). Therefore, it is necessary to examine whether their

spatial occurrence overlaps with each other at the spatial scales smaller than reported before even within a scale of several tens to one hundred meters (i.e. defined as river reach scale).

Few studies have shown co-occurrence distributions of the two pearl mussel species at the river reach scale in a wide geographical area. Therefore, this chapter aimed to show the regions where the two pearl mussel species potentially co-occur with a high possibility at the river reach scale (50–100 m) in Hokkaido, northern Japan. The species identification of the two pearl mussel species of Japan has been conducted using the differences of shell morphologies such as outer-shell characteristics (e.g. Akiyama et al. 2014). However, the identification of these two species using shell morphologies is often difficult because the appearances of these two species are very similar to each other. In addition, outer-shell morphologies can be variable among the habitat environments and/or regions (Takeuchi et al. 2007). Therefore, we relied on DNA analysis because it is recognized as one of the most powerful tools to overcome these problems (e.g. Campbell et al. 2008)

2-2. Methods

Study sites and sampling

Mussel sampling was conducted from 53 rivers across 31 watersheds in Hokkaido, northern Japan, where the distributions of pearl mussels have been confirmed by the present author and colleagues (Fig. 2-1). The studied rivers were divided into four geographical regions, east, north, center, and erimo (Figs. 2-1b, c). The breakdown of these rivers was as follows: 31 rivers across 17 watersheds in the east, 16 rivers across 8 watersheds in the north, 5 rivers across 5 watersheds in the center, and 1 river across 1 watershed in the erimo. Study rivers didn't exist in other regions such as Shiretoko and Oshima Peninsula because of the lack of information on recent mussel distributions. Mussels were collected from May 2016 to October 2017, but the mussels from one river in the east were collected on 29th September 2014. The detailed locations of the study streams remain undisclosed to aid the conservation of the endangered species. A 50–100 m study reach was established in each river. 1–3 investigator(s) collected 4–47 mussels using glass-bottomed viewing buckets from at least two separate patches of mussels in each study reach. The location of each study reach was recorded using a handheld GPS. The collected mussels were taken back to

the laboratory. In the study segment where mussel abundance was low (< c.a. 500 individuals), the investigator clipped the mantle tissue (< c.a. 1.5 mg) through the gently opened valves in the field, and immediately released them back to the study segment. The mantle clipping method has been previously used as a less destructive method for ecological and genetic studies of endangered freshwater mussels (e.g. Inoue et al. 2014). Collected tissue samples were preserved in 99.5% ethanol and stored at −20°C until DNA extraction for species identification.

Species identification through DNA extraction, polymerase chain reaction (PCR), and electrophoresis

Kurihara (2005) developed the identification protocols of the two target species using the gel electrophoresis banding patterns of 16S rRNA polymerase chain reaction products. Mitochondrial DNA is typically maternal inheritance. Species identification protocols using mitochondrial DNA can be sometimes unavailable due to the hybridization and introgression between related species. However, previous studies have shown that my two target species were genetically isolated from each other even when co-occurring the two species in the same stream (Kurihara et al. 2005). Therefore, the identification protocols developed by Kurihara (2005) were chosen to accurately and less harmfully identify two pearl mussels in this chapter.

The two pearl mussel species were discriminated by the gel electrophoresis banding patterns of the PCR products using four DNA primers on the mitochondrial 16S rRNA gene (Kurihara 2005, Miura et al. 2019a). While 16sar-L-myt (5′-CGACTGTTTAACAAAAACAT-3′) and 16sbr-H-myt (5′-CCGTTCTGAACTCAGCTCATGT-3′) (Lydeard and Davis 1996) amplify DNA fragments of both species (i.e. positive control), MRA16s01(5′-TGAATGGCAACACGGAAGAT-3′) and MRB16s01 (5′-GGGGCAACCCTGGAACCGTT-3′) are specific to *M. laevis* and *M. togakushiensis*, respectively (Kurihara 2005). The positive control, i.e. PCR products of 16sar-L-myt and 16sbr-H-myt, appears near 550 base pairs (bp). The length of two species-specific primers, MRA16s0l (*M. laevis*) and MRB16s01 (*M. togakushiensis*) are 350 bp and < 300 bp, respectively, which allows for rapid species identification (Fig. 2-2). Thus, the two species can be identified by observing the electrophoresis patterns of the amplified fragments (Kurihara 2005, Miura et al. 2019a).

Total genomic DNA was extracted from small pieces of mantle or muscle tissues (< c.a. 0.5 mg) using a Gentra Puregene Tissue Kit (Qiagen Corp., Hilden, Germany) following the manufacturer's protocol. PCR amplification using the four primers described above was performed in a 6 μL volume containing 0.2 μL ultrapure water, 3 μL Go Taq Colorless Master Mix (Promega Corp., Wisconsin, USA), 0.9 μL 16sbr-H-myt primer (5 pmol/μL), 0.3 μL of each primer (5 pmol/μL each), and 1 μL of the DNA template. The thermal profile of the PCR included the first step (a pre-cycling denature, 95°C, 2 min), 25 cycles of the second step (denature, 95°C, 45 s; annealing, 55°C, 1 min, extension, 72°C, 30 s), and the last step (extension, 72°C, 5 min). Amplified PCR products were electrophoresed on a 1.5% agarose gel and stained with Midori Green DNA Stain (NIPPON Genetics Co., Tokyo, Japan) for band characterization. The identifications of the two species were tested from the electrophoresis patterns of the amplified fragments.

Mapping

Three types of distributions, 1) areas where the two target species co-occurred in the same reach, 2) areas where *M. laevis* occurred, and 3) *M. togakushiensis* occurred, were separately charted on a 10 × 10 km^2 mesh map each. Each 10 × 10 km^2 mesh was categorized into three types in different symbols as follows: a) those not studied, b) those containing the studied reaches but no target species was found, and c) those containing the studied reaches and the target species was found, in each type of map. The meshes categorized *b* or *c* were referred to as "study mesh" hereafter. There was a total of 38 study meshes on each map. The breakdown of these meshes in each region was as follows: 21 meshes in the east, 11 meshes in the north, 5 mesh in the center, 1 mesh in the erimo (Figs. 2-1b, c). The Arc GIS 10.6 (ESRI inc., Redland. CA, USA) and Q GIS 2.18.14 (Quantum GIS Development Team 2016) were used for mapping of the distributions.

2-3. Results

A total of 1025 tissue samples were collected from the 53 rivers across the 31 watersheds, and 405 and 620 individuals were identified by DNA analysis as *M. laevis* and *M. togakushiensis*, respectively. The two target species co-occurred in 19 meshes of 38 study meshes (50%) from three regions except for the erimo (Fig. 2-3). The percentage of study meshes in which two mussels co-

occurred was highest in the east at 67% (14 of 21 study meshes). Subsequently, the percentages of study meshes in which two mussels co-occurred were 36% in the north (4 of 11 study meshes), 20% in the center (1 of 5 study meshes), and 0% in the erimo (0 of 1 study meshes) (Fig. 2-4a). In addition, the two target species co-occurred in 26 of 53 study rivers (49%). The percentage of study rivers in which two mussels co-occurred was highest in the east at 68% (21 of 31 study rivers). Subsequently, the percentages of study rivers in which two mussels co-occurred were 25% in the north (4 of 16 study rivers), 20% in the center (1 of 5 study rivers), and 0% in the erimo (0 of 1 study rivers), which was very similar patterns with the results of study meshes (Fig. 2-4b).

Margaritifera laevis was found in 29 of 38 study meshes (76%) (Fig. 2-5a). The percentages of study meshes in which *M. laevis* was found were 71% in the east (15 of 21 study meshes), 91% in the north (10 of 11 study meshes), 80% in the center (4 of 5 study meshes), and 0% in the erimo (0 of 1 study mesh). In addition, *M. laevis* was found in 39 of 53 study rivers (74%). The percentages of study rivers in which *M. laevis* was found were 71% in the east (22 of 31 study rivers), 81% in the north (13 of 16 study rivers), 80% in the center (4 of 5 study rivers), and 0% in the erimo (0 of 1 study river). *Margaritifera togakushiensis* was found in 29 of 38 study meshes (76%) (Fig. 2-5b). The percentages of study meshes in which *M. togakushiensis* was found were 100% in the east (21 of 21 study meshes), 45% in the north (5 of 11 study meshes), 40% in the center (2 of 5 study meshes), and 100% in the erimo (1 of 1 study mesh). In addition, *M. togakushiensis* was found in 40 of 53 study rivers (75%). The percentages of study rivers in which *M. togakushiensis* was found were 97% in the east (30 of 31 study rivers), 44% in the north (7 of 16 study rivers), 40% in the center (2 of 5 study rivers), and 100% in the erimo (1 of 1 study river).

2-4. Discussion

The study in this chapter showed the regions in Hokkaido where two endangered freshwater pearl mussels co-occur in the same study reach with high probability. Co-occurrences of the two pearl mussels in the same river reach were found from three regions (east, north, and center). The ratio of the number of study rivers where both species were found to the total number of study rivers was highest in the east (68%) among four study regions. Previous studies have fragmentarily shown the co-occurrences of two target species in the same rivers in eastern

Hokkaido (Machida and Akiyama 2013, Sakai et al. 2017), without a clear description of the spatial scale. As with these previous studies, my results support species distribution that the two target species likely to co-occur in the same study reach in eastern Hokkaido. In addition, my results also showed that there were also some possibilities of the co-occurrences of the two target species in the north and center although the possibilities were not as high as in the east.

The distributions of the two pearl mussels have been previously shown at the scales larger than that examined in this chapter (i.e. several to several ten meters) (e.g. Takeuchi et al. 2007, Sakai et al. 2017). These results provide valuable information for effective conservation and management of the two species when large-scale changes occur in cases such as the establishment of the protected areas and the development of large dams. The information on the co-occurring distributions of the two species at the river reach scale (several to several hundred meters) also provides valuable information for the management of river environments. For example, river improvement works (e.g. excavation of the riverbed, widening of the river width, and consolidations of the riverbank) have been often conducted at the river reach scale. In such cases, knowledge of co-occurrences of the two pearl mussels can help to make decisions in which species and/or which host fish (*O. masou masou* or *S. leucomaenis leucomaenis*) should be targeted in conservation efforts.

The spatial map results must be viewed with caution. From the points of aiding the conservation of the two species, 10 × 10 km^2 mesh maps were used for mapping the results in this chapter. However, these results couldn't exactly show where is the investigated river reaches in the maps because the scale of meshes is larger than the actual scale of the study reaches (i.e. 50–100 m). Regardless of this discrepancy of the spatial scale, the present results can enhance attentions for managers and investigators to enough pay attention to the regions with high possibilities of co-occurrences of the two pearl mussels when planning the conservation measures and investigations. It will be more effective if they can additionally collect the information on the target species such as the ratio of the two pearl mussels inhabiting the target river reach.

The present results showed that eastern Hokkaido is a region with high possibilities of co-occurrences of the two pearl mussels in the same study reach. Although the mechanism behind this pattern is unclear, and this is a topic for future research, I propose two hypotheses about the

mechanism. First, preferred habitats of adult individuals (including small immature individuals) of each species overlap in some areas but not in some other areas. For example, presuming that maximum temperature limiting their fitness is different, distributions of both species may not be limited by water temperature in the middle-lower rivers in eastern Hokkaido where the maximum temperature is naturally low because of weather conditions being well below the threshold level of both species (e.g. 20-degree Celsius that is the general maximum suitable temperature for both species, Kondo 2008). Therefore, they co-occur in those rivers but not necessarily in rivers in other regions where water temperature reaches the threshold level for one species. Second, the spatial distributions of mussels are controlled by the distributions of host fish. The distributions of freshwater mussels including margaritiferids can be limited by the distributions of host fish because they require parasitization on host fish during their larval stage (Vaughn and Taylor 2000). For example, *S. leucomaenis leucomaenis* generally distributes in more upstream than *O. masou masou* (e.g. Morita et al. 2016). However, this longitudinal distribution pattern is often vague in eastern Hokkaido (e.g. Nagasawa et al. 2009). As a result of these examples, the species-specific distributions of the two pearl mussels can be more blurred in eastern Hokkaido.

The co-occurring distributions of the two pearl mussels were not found from the erimo among the four study regions. However, it is difficult to infer the co-occurrences of the two species are unlikely to happen in the erimo due to the small numbers of the study rivers. The present results only showed the distribution of *M. togakushiensis* in the erimo (Figs. 2-5a, b) while the previous study only found the distribution of *M. laevis* in the erimo (Kondo 2008). It is unclear whether my study river in the erimo is same as the river investigated in Kondo (2008). However, the three potential reasons for the discrepancy can be raised. First, the study in this chapter was unable to find *M. laevis* in this region although it was present perhaps because of low abundance or presence in other rivers. Second, it was the case in Kondo (2008) for *M. togakushiensis*. Third, Kondo (2008) misidentified *M. togakushiensis* as *M. laevis*. Furthermore, the study did not investigate distributions in the Oshima Peninsula, Rebun Island, and Minami-Furano town (Fig. 2-1b), where the distributions of margaritiferids have been previously found (Okada and Kiba 1931, Kondo and Kobayashi 2005). Further research is needed in these regions to clarify the co-occurrence distributions of the two pearl mussels in the future.

The species identification of the two pearl mussels was conducted using DNA analysis in this chapter instead of identification criteria using shell morphologies that have been previously used (Kondo and Kobayashi 2005, Akiyama 2007, Awakura et al. 2009). DNA analysis enabled me to accurately distinguish the two pearl mussels. However, species identification using DNA analysis like this chapter is not cost- and time- effective when targeting a number of mussel individuals in the field. In Japan, the investigators of private companies and individual researchers are often difficult to access the technical equipment and have limitations of time and financial budgets in many cases in environmental assessments. The study in this chapter showed that two pearl mussel species can easily sympatrically distribute at a relatively smaller spatial scale than before reports, i.e. river reach scale. It indicates that the importance of accurate and more cost- and time-effective species identification is needed to properly investigate and/or plan conservation measures of two pearl mussels. Therefore, it is preferable to develop a methodology to cheaply and quickly identify them without running DNA analyses which were described in the next chapter.

The study in this chapter showed that the potentialities of co-occurring distributions of the two pearl mussels among four study regions in Hokkaido, northern Japan, and these two species can also co-occur in three study regions except for the erimo (i.e. east, north, and center). These results of the co-occurrence distributions of the two pearl mussels might provide important information for the conservation and management of the two species. It can help to decide whether the co-occurrences of the two species should be taken into consideration when conducting the investigation and/or conservation practices. However, the study in this chapter couldn't assess the population status of the target species such as mussel density, age distribution, and recruitment. The determining of the detailed population status is imperative for clarifying the cause of the population decline of *M. togakushiensis* as stated in Chapter 1. To do so, many mussel individuals from small immatured to large matured individuals should be accurately distinguished without lethality when determining such detailed population status of the two species especially in regions with high possibilities of co-occurrences of the two target species clarified in this chapter (e.g. eastern Hokkaido). In order to the achievement of the aim of this thesis, the nonlethal identification criteria of the two pearl mussels should be established in Chapter 3.

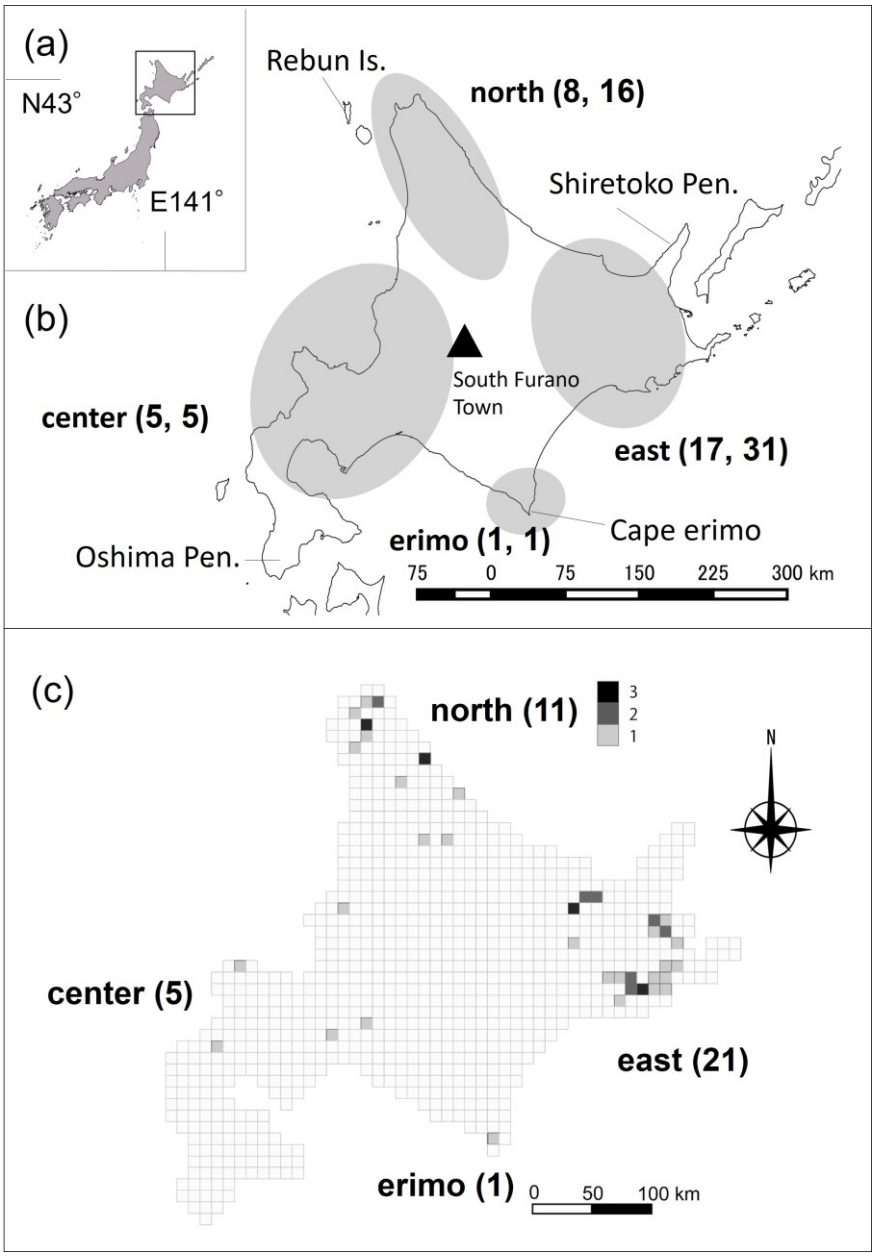


Fig. 2-1 The geographical location of Hokkaido (a) and study area with the name of each region (b), and 10 × 10 km mesh map with the number of study meshes (c). The ellipses in b mean the area containing study rivers with the number of study watersheds and rivers in the brackets respectively. The triangle in b indicates the location of South Furano Town. The differences in color of meshes indicate the number of the study river contained, and white means that there were no study rivers (reaches)

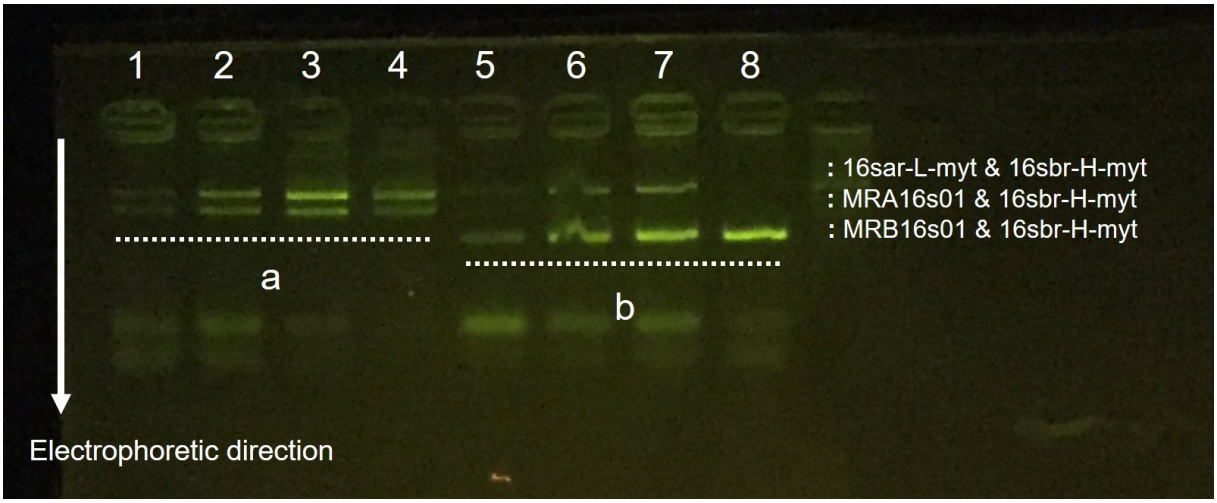


Fig. 2-2 An example of the gel electrophoresis banding patterns of the polymerase chain reaction (PCR) products using four DNA primers on the mitochondrial 16S rRNA gene of *M. laevis* (a) and *M. togakushiensis* (b). Each band corresponds to the combination of each PCR primer. The arrow indicates the direction of the electrophoresis

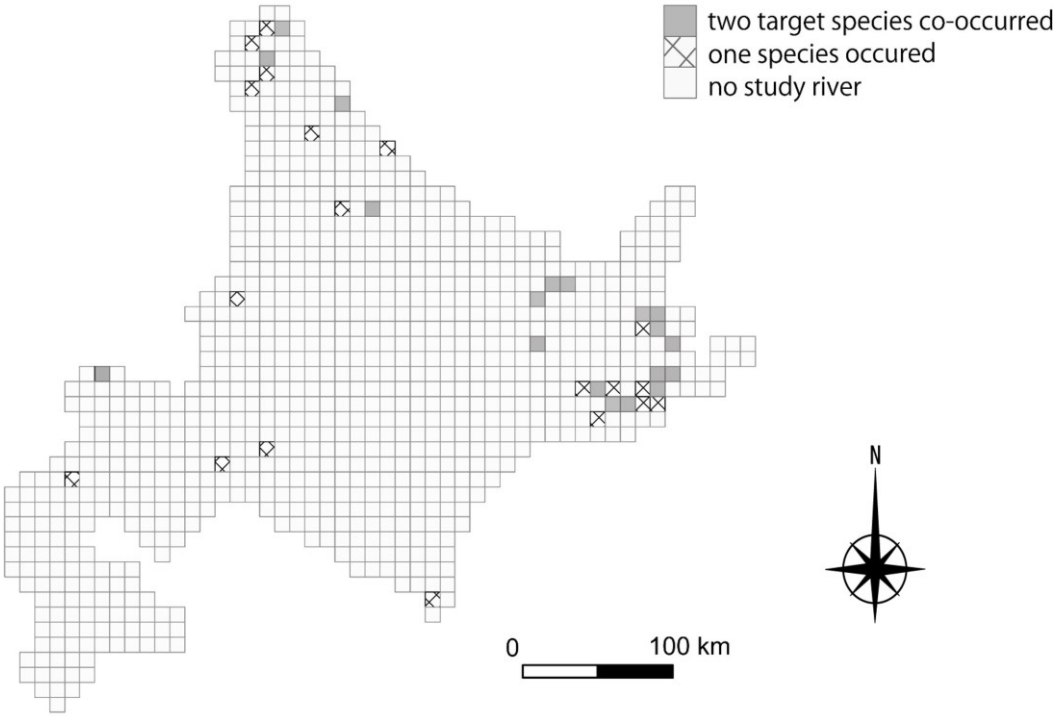


Fig. 2-3 The geographical locations of the study meshes containing the rivers where the co- *M. laevis* and *M. togakushiensis* co-occurred (gray), each species occurred (shade), and study rivers were not contained (white). The size of each mesh is 10 ×10 km^2

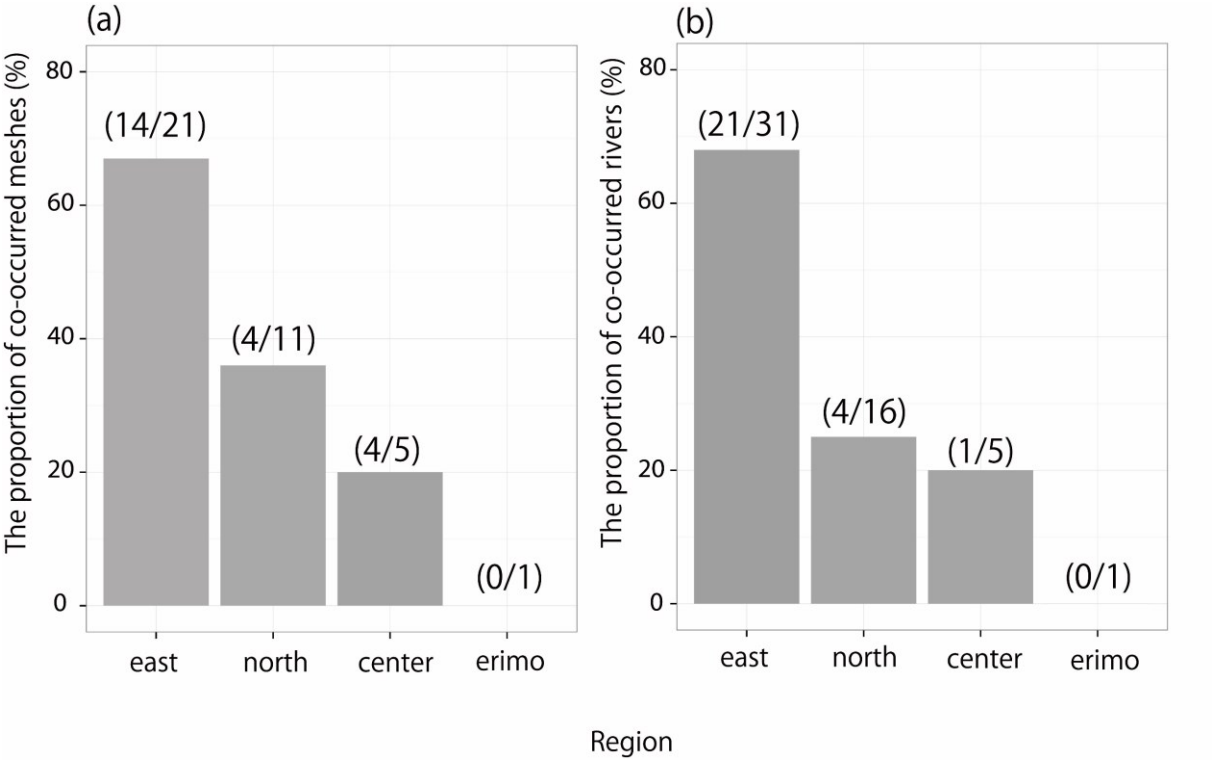


Fig. 2-4 The proportion of the number of meshes (a) and rivers (b) where the *M. laevis* and *M. togakusiensis* co-occurred to the total number of study meshes in each region. The numbers on bars in brackets means the total number of rivers in each study region (right) and rivers where co-occurrence distributions were confirmed (left), respectively

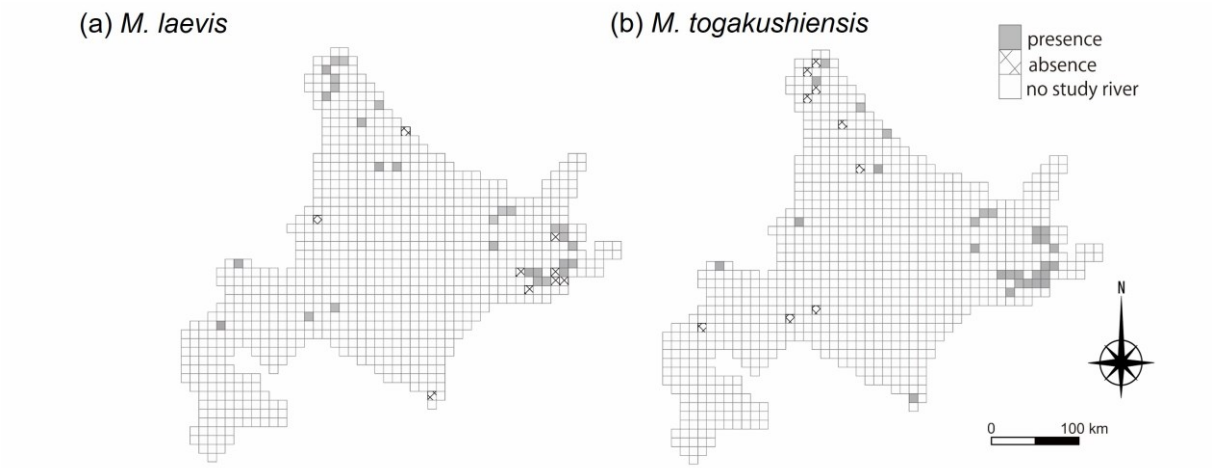


Fig. 2-5 The geographical location of the meshes where *M. laevis* (a) and *M. togakushiensis* (b) occurred. The differences in mesh patterns mean the meshes where the target species was the presence (gray), absence (shade), and study rivers were not contained, respectively. The size of each mesh is 10 ×10 km^2

3. Simple non-lethal identification criteria for two endangered freshwater pearl mussels, *Margaritifera laevis* and *M. togakushiensis*, in Hokkaido, northern Japan

3-1. Introduction

Species identification is a fundamental process for ecological studies and conservation practices. Misidentification potentially causes data bias, which produces incomplete and/or false results (Royle and Link 2006, Fitzpatrick et al. 2009, Shea et al. 2011). Academic experts have developed accurate species identification techniques (e.g. observation of external and/or internal morphology or DNA analysis), which are not always readily accessible to the general public. The importance of ecological data collected by the general public for the conservation of wildlife has become increasingly recognized because there is the potential for spatiotemporally extensive data sets to be obtained (so-called "citizen science," e.g. Cohn 2008, Bonney et al. 2009, Devictor et al. 2010). The accuracy of sophisticated species identification depends on the observer's experiences and/or financial budget (Sauer et al. 1994, Lotz and Allen 2007, Shea et al. 2011), which hinders the potentiality of citizen science. Therefore, there is a great demand for identification methods and criteria that are simple, cost-effective, and not affected by inter-observer variability.

Recent studies have shown that one of the most important problems for mussel conservation is recruitment failure. Mussel populations without recently born juveniles will become extinct in the near future, even though the current size of the populations might be relatively large (Österling et al. 2008, Geist 2010, Ferreira-Rodríguez et al. 2019). Thus, long-term monitoring targeting a wide age class of mussels is required. Some species have been recently targeted in citizen-led monitoring programs and conservation practices (Miura et al. 2014, 2018a, Estuary 2018). Their imperiled status thus necessitates only non-destructive (i.e. non-lethal and least harmful to individuals) monitoring methods.

Species identification of freshwater mussels has been traditionally conducted by shell morphology (e.g. length, height, width, and sculptures) (Strayer and Smith 2003). However, morphology-based species identification has its limitations because shell morphologies sometimes exhibit intra-specific variations that are influenced by ambient environments and life-history stages (Eagar 1978, Zieritz and Aldridge 2009, Inoue et al. 2013, Zajac et al. 2018). Some species must be

sacrificed because identification keys are based on inner-shell morphologies such as the pseudocardinal and adductor muscle scar (Shea et al. 2011). As applied to the study in Chapter 2, DNA-based identification is recognized as one of the most powerful tools to overcome these problems (e.g. Campbell et al. 2008, Boyer et al. 2011), which can provide an accurate identification with a small amount of tissue (e.g. Inoue et al. 2013). However, the use of DNA-based identification for ecological monitoring by non-experts is limited by the costs involved and expertise required. One of the best solutions is to develop shell morphology-based identification criteria that are robust to intra-specific morphological variations, with the accuracy validated by DNA analyses. However, few studies have previously developed non-lethal morphology-based species identification criteria of freshwater mussels with a wide range of sizes because most studies have focused on relatively large individuals (e.g. matured individuals) (Kondo et al. 2011).

Freshwater pearl mussels, *Margaritifera laevis* and *M. togakushiensis* typically have maximum lengths of less than 150 and 100 mm, respectively (Kondo 2008). Both species inhabit gravelly to sandy cold-water rivers, and occasionally co-occur within the same river segments (Kondo 2008). In addition, two pearl mussel species can also co-occur at the same river reaches (i.e. 50–100 m) as shown in Chapter 2. They possess similar appearances including the absence of shell-surface sculptures (Fig. 3-1) and *M. togakushiensis* was only recently classified as a separate species (Kondo and Kobayashi 2005). The misidentification of these two species could lead to different conservation approaches because of the major differences in their reproductive timing and host salmonid fish (Kurihara 2005, Kondo and Kobayashi 2005, Kobayashi and Kondo 2009, Kurihara and Goto 2011, Terui et al. 2015). The practical use of available identification keys, such as the scar shape of the adductor muscle (Kondo and Kobayashi 2005), the morphology of post-parasitic juveniles (shell length: 300–600 μm) (Kobayashi and Kondo 2007), and the shell morphology of large individuals (shell length: > 47 mm) measured in two rivers (Kurihara et al. 2005) in citizen science is limited.

To improve species identification of two *Margaritifera* species, this chapter attempted to establish simple and non-lethal identification criteria for *M. laevis* and *M. togakushiensis* using both external morphology and DNA analysis. The specific objectives were 1) to examine key morphological features that distinguish the two species and 2) to establish a simple linear

discriminant function (LDF) of small and large individuals (i.e. mature and immature individuals) for the two pearl mussels by considering regional morphological differences. The study first identified field-collected individuals based on their DNA, from which shell morphological keys distinguishing them were established. The study targeted individuals in the area encompassing a total of > 30,000 km^2 in Hokkaido, northern Japan and analyzed individuals having a wide range of sizes (shell length: 6.7–132.8 mm).

3-2. Methods

Study region

The study in this chapter conducted in 52 rivers in Hokkaido, Japan, where the distributions of pearl mussels have recently been investigated in Chapter 2 (Miura et al. 2019a). I divided the study rivers into two geographical regions with the Hidaka and Daisetsu Mountains as the center: "east" and "west" (Fig. 3-2) because I previously found that the shell height of *M. togakushiensis* in the east was visually higher than those in the west (identification was conducted using DNA analyses; Miura et al. 2019a). The east and west regions had a total of 28 rivers composed of 16 watersheds and 24 rivers of 16 watersheds, respectively. The detailed locations of the study streams remain undisclosed owing to conservation considerations.

Field sampling

I collected mussels from the years of 2014 to 2018 (N = 1091): 1025 individuals were also used in Chapter 2 and the remaining 66 individuals were additionally collected to make up for a lack of small size individuals (< 20 mm length) in 2018. I established a 50–100 m study segment in each river, and 1–3 investigator(s) collected mussels [1–76 (mean: 21) individuals/river] using glass-bottomed viewing buckets from at least two separate patches of mussels (except for one river where only one individual was collected). The collected mussels were taken back to the laboratory and each was measured for three shell morphological characteristics [length (anterior to posterior), height (dorsal to ventral), and width (right to left valve)] to the nearest 0.1 mm using calipers (Fig. 3-3), followed by the removal of the mantle or foot muscle tissues (N = 957). In the study segment where mussel abundance was low (< c.a. 500 individuals), I measured shell morphology, clipped

the mantle tissue (< 1.5 mg) through the gently opened valves in the field, and immediately released them back to the original study segment (N = 131). The mantle clipping method has been used previously as a less destructive method for ecological and genetic studies of endangered freshwater mussels (e.g. Inoue et al. 2014). Collected tissue samples were preserved in 99.5% ethanol and stored at −20°C until DNA extraction. In addition, I used specimens from a museum (N = 19) collected between 2008 and 2013 (Table 3-1).

Species identification

Species identification was conducted as with the methods of DNA-analysis in Chapter 2. I used the identification protocols established by Kurihara (2005), which observes the gel electrophoresis banding patterns of 16S rRNA polymerase chain reaction products. The detailed materials and protocols were described in Chapter 2.

Statistical analyses

Linear discriminant function (LDF) was used to examine the key morphological differences and to establish identification criteria between the two species. LDF has been used to reveal the morphological differences of two or more freshwater mussel species (Kennedy and Haag 2005, Beggel et al. 2015). In addition, LDF has two advantages for establishing shell morphology-based identification criteria. First, constructed function(s) can classify the newly collected individuals as species categories using morphological factors. Second, LDFs are described as simple linear equations. Thus, users can easily classify the samples by substituting the factors to the equation. Morphological characteristics of freshwater mussels can differ between mature and immature individuals (Haag 2012). Therefore, I classified samples into two size classes based on their shell length and constructed LDFs for each: smaller than 40 mm and equal to or larger than 40 mm. The threshold size (40 mm) was set as the smallest size of sexual maturity for both species (Akiyama 2007; K. Miura personal observation). First, I constructed LDFs using the raw morphological data (length, height, and width) as explanatory variables and species as a response variable. Next, I constructed LDFs using ratios (height/length, width/length, and width/height ratio) as explanatory variables and species as a response variable. The ratio data has been widely

used for comparing intra-specific morphological differences of freshwater mussels because the ratio data can standardize the variables of size. For raw data functions, I only used 40–100 mm *M. laevis* individuals for the LDFs to set the size ranges for the two species that were comparable because the maximum length of *M. laevis* was larger (see Results section in this chapter). Forward stepwise procedure was performed for each case to determine the best LDFs that contained the sets of variables and coefficients with the lowest error rates for differentiating the two species. To test whether the differentiated species groups significantly differed in morphological characteristics, raw or ratio data were compared among groups for each best LDF. I applied multivariate analysis of variance (MANOVA) when multiple variables remained in best LDFs whereas one-way ANOVA was used for best LDFs that contained single variables. The constructed LDF(s) can estimate the species category using the differences of the signs of LD score (positive or negative). Thus, I also calculated the mean LD score of each species to determine the sign of the LD score of each species.

I then established criteria for the two species by considering regional morphological differences. Based on the above selected morphological variables, I constructed LDFs and calculated an identification accuracy [(number of correctly identified individuals/total number of individuals examined) × 100] of each function for the following cases; 1 the functions constructed for individuals of both regions were applied to individuals of each region, and 2) the functions constructed for individuals of each region were applied to individuals of each region. From the results of the analysis of the above two cases I then separately constructed the final LDFs for each of the two regions, using raw and ratio data. Accuracies of the final LDFs were assessed for each of the five-length classes (0–20, 20–40, 40–60, 60–80, and 80–100 mm; > 100 mm length only for *M. laevis*). All statistical analyses were conducted using R version 3.0.3 (R Development Core Team 2015) with the significant level of $\alpha = 0.05$. LDF and variable selection were computed using MuMIn (Barton 2011) and klaR (Roever et al. 2018) packages of R.

3-3. Results

Among the 1110 individuals included in the present study, 481 and 629 individuals were identified by DNA analysis as *M. laevis* and *M. togakushiensis*, respectively. *Margaritifera laevis* and *M. togakushiensis* occurred in both regions, and the breakdown of these was as follows: 207

individuals of *M. laevis* and 486 individuals of *M. togakushiensis* from the east, and 274 individuals of *M. laevis* and 143 individuals of *M. togakushiensis* from the west.

Interspecific morphological differences

The maximum shell length differed between the two pearl mussel species. *Margaritifera laevis* was over 100 mm in length and *M. togakushiensis* was less than 100 mm (Table 3-2). Forward stepwise procedures selected best LDFs with length and height, and with height/length as explanatory variables regardless of the size classes (Table 3-3, Figs. 3-4 and 3-5). LDFs using individuals collected from both study regions revealed significant morphological differences between *M. laevis* and *M. togakushiensis* for all size classes (MANOVA and one-way ANOVA, $p < 0.01$ for each). The mean LD scores of *M. laevis* were negative and those of *M. togakushiensis* were positive (Table 3-3).

Accuracy of discriminant functions

The accuracies of LDFs in the east region were higher than those in the west region in the cases where LDFs constructed based on individuals from both regions were applied to each region (Table 3-4). The accuracies of LDFs tended to be higher in the cases when LDFs constructed for each region were applied to each region than in the cases where LDFs constructed for both regions were applied to each region (Table 3-4). The height-to-length ratio of *M. togakushiensis* was higher in the east than in the west, whereas that of *M. laevis* did not differ between the two regions (Table 3-2, Fig. 3-6). I separately constructed the final LDFs for each east and west region, using raw and ratio data (Table 3-5). The final LDFs identified two species with good accuracy: 85–96% accuracy in the east and 67–96% accuracy in the west (Table 3-6). The accuracies of the final LDFs between the raw and ratio data were more similar to each other with an error of ± 6% in the east compared to the west where maximum differences of ± 14% were found. These functions identify individuals as *M. laevis* when the solution is positive, and *vice versa*. In addition, I found that individuals > 100 mm in length can be perfectly identified as *M. laevis* regardless of region studied.

3-4. Discussion

The study in this chapter developed simple and non-lethal identification criteria for *M. laevis* and *M. togakushiensis*, using both external morphology and DNA analyses. From the samples with a wide size range collected over a broad geographical range, I found that 1) the maximum shell length of *M. laevis* was greater than 100 mm and of *M. togakushiensis* was less than 100 mm, 2) the morphological differences of the two species were best characterized by length and height (or their ratios) regardless of size class, with *M. laevis* being elliptically elongate (smaller height/length) and *M. togakushiensis* being ovately elongate (larger height/length), and 3) there was a regional morphological difference, especially for *M. togakushiensis*. In addition, I established LDFs with high identification accuracies for the two species in each study region and shell-size class. Increased levels of identification accuracy when considering regions suggests that these two pearl mussel species can be better identified by considering geographical variations in shell morphology. I also found that the LDFs using simple morphological raw data (i.e. LDFs in Table 3-5a) almost equally or better explained the difference between the two species compared with LDFs using ratio data (mean accuracies of raw vs ratio data, east: 92.0% vs. 92.6% and west: 82.6% vs. 78.6%). Thus, I recommend the use of LDFs with raw data. A summary of the simple usage of established identification criteria is shown in Figure 3-7.

The previous identification criterion for the two pearl mussel species was based on a lethal technique using the scar shape of the adductor muscle in the shell (e.g. Takeuchi et al., 2007). My identification criteria are non-destructive; therefore, will enable conducting ecological studies without increased pressures on dwindling populations of endangered species. I also found that the LDFs using simple morphological data, length and height, could explain the difference between the two species. These morphological characteristics can be measured by people who have not received intensive training and with limited experience, expertise, and expensive equipment. Thus, I believe that the identification criteria will be broadly applicable to citizen science and monitoring as well as academic studies. These morphological measurements can be quickly conducted by taking digital photographs of individuals with their sides of shells horizontally placed on a flat surface, and later processing with image analysis software (e.g. Image J software, Negishi et al. 2018). Minimized handling time will reduce overall handling stress for individuals and may also contribute to efficient species identification.

My findings on morphological characteristics of the two pearl mussels generally coincide with previous findings from limited study regions (1–2 rivers) and narrow ranges of shell size (Kurihara et al. 2005, Kobayashi and Kondo 2007). The shell morphological characteristics of immature individuals (6.7–40.0 mm shell length) were also coincident with general morphological patterns of the two species. The majority of previous studies examining the morphological characteristics of freshwater mussels have focused on limited life-history stages (i.e. mature individuals and glochidium larvae) (Kennedy and Haag 2005, Kurihara et al. 2005, Kondo et al. 2011). However, the ecological characteristics of freshwater mussels such as distribution or movement patterns can differ between small and large individuals (Hastie et al. 2000a, Negishi et al. 2011). In addition, the juvenile stage is considered to be the most sensitive life-history stage (Österling et al. 2008, Strayer and Malcom 2012). The lack of a young age class in a mussel population is an indication of a high risk of future extinction due to zero recruitment (Haag 2012, Ferreira-Rodríguez et al. 2019). Therefore, the proposed criteria would contribute to a comprehensive understanding of mussel ecology and population status.

Another new finding of the study was that the shell morphologies differed regionally. Kurihara et al. (2005) showed that the mean height-to-length ratio of three *M. laevis* populations was 0.48, and that of a *M. togakushiensis* population was 0.53 (excluding a population where only two individuals were collected). These ratios of mature *M. laevis* matched the present results in both regions; however, the ratios of mature *M. togakushiensis* in my study were higher in the east (mean: 0.55) and lower in the west (mean: 0.51) than the values in Kurihara et al. (2005). This regional morphological difference might be the reason for the variable identification accuracy of *M. togakushiensis* between regions, especially for the individuals of > 40 mm in size. Shell morphologies of freshwater mussels are variable among regions and habitat owing to the differences in environmental factors such as substrate, velocity, and depth (ecophenotypic plasticity, e.g. Hornbach et al. 2007, Zieritz and Aldridge 2009, Inoue et al. 2013, Zajac et al. 2018). Further studies are required to understand the drivers of the regional morphological differences of *M. togakushiensis*, which would contribute to the development of better identification criteria using shell morphologies.

My identification criteria should be carefully used based on the purposes of research or

monitoring programs. First, my LDFs can be applied as powerful tools for the identification of the two pearl mussel species for where mussel abundance or age structure is assessed in natural populations. On the other hand, the established LDFs potentially have misidentification with 4%–14% (mean: 8%) in the east and 4%–33% (mean: 17%) in the west in accuracy except for the ≥ 100 mm sized individuals (accuracy: 100%) even though the overall accuracy was high. Therefore, when the determination of the occurrence (i.e. presence or absence) of *M. laevis* is needed, a more accurate index such as maximum shell length is effective for judgments (i.e. the presence of ≥ 100 mm individuals strongly suggests the presence of *M. laevis*). Furthermore, I can also accurately judge the presence of *M. togakushiensis* by focusing on the specific size classes with higher identification accuracy (e.g. 20–40 and 60–80 mm in the east, 0–20 mm in the west). Second, the accuracies of my identification criteria are not necessarily ensured when the LDFs are applied to individuals collected from other regions (e.g. Sakhalin and Honshu). My field sampling did not cover all distribution ranges of the two species. The study in this chapter suggested that the shell morphologies of the two species could differ regionally in northern Japan. Therefore, it is difficult to judge whether my criteria are applicable to other regions from the study in this chapter. Testing the applicability of my identification criteria in other regions is a requirement for future studies. Third, the accuracies of LDFs could be somewhat variable under a variety of local environments owing to the ecophenotypic plasticity of freshwater mussels. I strongly believe that my criteria are applicable to a wide range of local environments because my LDFs were constructed by the individuals collected from more than two patches each in a total of 52 rivers. However, accuracies of LDFs would be variable under extreme environments such as fast current, deep water depth, and hard substrate (Haag 2012). Thus, users should carefully use my criteria under such situations. Even under the latter two limitations, previous studies have shown that the maximum shell length of *M. togakushiensis* is typically not over 100 mm in other regions and local collection points (Kurihara 2005, Kurihara et al. 2005, Kondo 2008). The difference in maximum shell length between the two species may be a good criterion for the judgment of the presence of *M. laevis*.

In conclusion, I established simple non-lethal identification criteria for two endangered freshwater pearl mussels. Several freshwater mussel species have been identified by lethal techniques or DNA analyses (Shea et al. 2011, Inoue et al. 2014). These methods are of limited use

when examining the population status of endangered species across a large area. Furthermore, the quantitative data of organisms (i.e. abundance) can generally be more informative for detecting the factors regulating the distributions in detail compared to presence/absence data (e.g. Randin et al. 2009). The improvement of identification techniques by academic studies like mine could open an avenue to the development of citizen science and monitoring as well as academic studies on such species. My identification method has an advantage over traditional methods such as keys concerning inner-shell morphology and DNA analyses because of its rapidness, simplicity, and cheapness. As with other freshwater mussel species, the target two species have been dramatically declining in recent decades (Ministry of the Environment of Japan 2014). However, the status of *M. laevis* and *M. togakushiensis* populations remain unclear, although effective management of both species is needed (Akiyama 2007, Miura et al. 2019a). Therefore, the detailed population status of *M. togakushiensis* determined using the new identification criteria in the next chapter. Ecological investigation of mussel abundance or age structure can help form a large-scale database on the population status of these two species.

Table 3-1 Pearl mussel specimens of the Bihoro Museum used in the present study. The unit of Length, Height, and Width is "mm". Locality of each specimen remains undisclosed for the conservation of endangered species

ID	Species	Sampling day	Length	Height	Width	Collector
BIHM0200067	*Margaritifera* sp.	05. Oct. 2008	71.3	36.9	22.6	Y. Machida
BIHM0200068	*Margaritifera* sp.	05. Oct. 2008	72.6	36.4	21.7	Y. Machida
BIHM0200073	*Margaritifera* sp.	13. May 2009	82.1	41.3	25.1	Y. Machida
BIHM0200074	*Margaritifera* sp.	13. May 2009	75.3	38.2	22.4	Y. Machida
BIHM0200075	*Margaritifera* sp.	13. May 2009	84.6	44.0	26.4	Y. Machida
BIHM0200076	*Margaritifera* sp.	13. May 2009	85.8	43.0	24.9	Y. Machida
BIHM0200077	*Margaritifera* sp.	13. May 2009	79.0	43.6	22.8	Y. Machida
BIHM0200078	*Margaritifera* sp.	13. May 2009	80.5	42.1	24.4	Y. Machida
BIHM0200079	*Margaritifera* sp.	13. May 2009	80.9	43.7	26.0	Y. Machida
BIHM0200080	*Margaritifera* sp.	13. May 2009	83.2	42.0	24.3	Y. Machida
BIHM0200081	*Margaritifera* sp.	13. May 2009	84.8	43.3	26.1	Y. Machida
BIHM0200082	*Margaritifera* sp.	13. May 2009	80.3	40.5	25.2	Y. Machida
BIHM0200320	*Margaritifera* sp.	18. June 2013	81.1	42.0	25.3	Y. Machida
BIHM0200324	*Margaritifera* sp.	18. June 2013	81.9	39.8	25.6	Y. Machida
BIHM0200325	*Margaritifera* sp.	18. June 2013	65.5	35.4	19.5	Y. Machida
BIHM0200326	*Margaritifera* sp.	18. June 2013	63.5	34.3	17.6	Y. Machida
BIHM0200327	*Margaritifera* sp.	18. June 2013	85.0	47.1	26.2	Y. Machida
BIHM0200328	*Margaritifera* sp.	18. June 2013	80.3	40.7	24.6	Y. Machida
BIHM0200329	*Margaritifera* sp.	18. June 2013	70.3	39.3	23.7	Y. Machida

Table 3-2 The number of specimens (N) and basic morphological characteristics of two pearl mussels of each region and size class. Each number means minimum-maximum values (mean ± SD). The unit of Length, Height, and Width is "mm"

Region/Species	Size	Length	Height	Width	Height/Length	Width/Length	Width/Height	N
(a) All area								
M. laevis	all	7.00–132.80 (71.38 ± 30.28)	4.10–57.40 (34.12 ± 13.64)	2.30–36.40 (20.39 ± 8.72)	0.41–0.60 (0.49 ± 0.03)	0.24–0.35 (0.29 ± 0.02)	0.48–0.70 (0.59 ± 0.04)	481
M. togakushiensis	all	6.70–96.80 (55.79 ± 17.99)	4.20–47.80 (30.11 ± 9.21)	2.50–28.70 (17.25 ± 5.54)	0.46–0.65 (0.54 ± 0.03)	0.24–0.39 (0.31 ± 0.03)	0.47–0.67 (0.57 ± 0.03)	629
M. laevis	≥ 40mm	40.20–132.80 (81.78 ± 21.80)	19.90–57.40 (38.90 ± 9.52)	10.40–36.40 (23.31 ± 6.48)	0.41–0.53 (0.48 ± 0.02)	0.24–0.34 (0.28 ± 0.02)	0.48–0.70 (0.59 ± 0.04)	397
	< 40mm	7.00–39.00 (22.27 ± 8.76)	4.10–19.80 (11.54 ± 4.32)	2.30–11.50 (6.58 ± 2.39)	0.47–0.60 (0.52 ± 0.02)	0.26–0.35 (0.30 ± 0.02)	0.51–0.67 (0.57 ± 0.04)	84
M. togakushiensis	≥ 40mm	40.20–96.80 (62.74 ± 11.16)	21.60–47.80 (33.72 ± 5.49)	11.00–28.70 (19.73 ± 3.49)	0.46–0.65 (0.54 ± 0.03)	0.24–0.39 (0.31 ± 0.03)	0.48–0.67 (0.57 ± 0.03)	511
	< 40mm	6.70–39.80 (25.90 ± 9.07)	4.20–23.00 (14.60 ± 4.93)	2.50–13.80 (8.11 ± 2.78)	0.53–0.63 (0.57 ± 0.02)	0.27–0.39 (0.32 ± 0.02)	0.47–0.65 (0.56 ± 0.03)	118
(b) East								
M. laevis	≥ 40mm	41.00–132.80 (88.33 ± 20.49)	20.70–57.40 (41.87 ± 8.83)	10.40–36.40 (25.59 ± 5.89)	0.41–0.53 (0.48 ± 0.02)	0.25–0.34 (0.29 ± 0.02)	0.49–0.70 (0.61 ± 0.04)	180
	< 40mm	9.80–37.20 (21.89 ± 6.88)	5.20–18.70 (11.25 ± 3.44)	3.40–11.20 (6.61 ± 1.95)	0.48–0.56 (0.51 ± 0.02)	0.26–0.35 (0.30 ± 0.02)	0.51–0.67 (0.59 ± 0.05)	27
M. togakushiensis	≥ 40mm	40.20–90.50 (62.42 ± 10.44)	21.60–47.80 (34.0 ± 5.40)	11.0–28.70 (19.63 ± 3.34)	0.49–0.65 (0.55 ± 0.03)	0.24–0.39 (0.31 ± 0.02)	0.48–0.65 (0.58 ± 0.03)	414
	< 40mm	6.70–39.80 (28.63 ± 8.68)	4.20–23.00 (16.13 ± 4.78)	2.60–13.80 (8.90 ± 2.78)	0.53–0.63 (0.57 ± 0.02)	0.27–0.39 (0.31 ± 0.02)	0.47–0.65 (0.55 ± 0.04)	72
(c) West								
M. laevis	≥ 40mm	40.20–125.90 (76.34 ± 21.41)	19.90–57.20 (36.43 ± 9.37)	10.80–36.30 (21.42 ± 6.34)	0.43–0.53 (0.48 ± 0.02)	0.24–0.33 (0.28 ± 0.02)	0.48–0.70 (0.58 ± 0.04)	217
	< 40mm	7.00–39.00 (22.45 ± 9.57)	4.10–19.80 (11.68 ± 4.70)	2.30–11.50 (6.57 ± 2.59)	0.47–0.60 (0.53 ± 0.02)	0.26–0.35 (0.30 ± 0.02)	0.51–0.62 (0.57 ± 0.03)	57
M. togakushiensis	≥ 40mm	40.50–96.80 (64.11 ± 13.81)	22.20–46.60 (32.49 ± 5.73)	11.70–27.40 (18.28 ± 3.73)	0.46–0.60 (0.51 ± 0.03)	0.24–0.37 (0.29 ± 0.02)	0.51–0.67 (0.56 ± 0.03)	97
	< 40mm	7.50–38.70 (21.48 ± 8.04)	4.40–21.30 (12.21 ± 4.19)	2.50–11.80 (6.89 ± 2.31)	0.53–0.62 (0.57 ± 0.02)	0.28–0.37 (0.32 ± 0.02)	0.51–0.61 (0.57 ± 0.02)	46

Table 3-3 Summary of the results of the linear discriminant functions (LDFs) with selected variables and statistical significances tested using multivariate analysis of variance (MANOVA) or one-way ANOVA of each size class

Predictor	Size class/Species	Mean LD score	Accuracy	Linear discriminant function	F (d.f.)	*p*
length, height	40-100 mm					
	M. laevis	−1.24	85%	y = − 0.27 × (length) + 0.53 × (height) + 0.07	739.3 (1) *	< 0.01
	M. togakushiensis	0.76				
	< 40 mm					
	M. laevis	−1.39	93%	y = −1.39 × (length)+ 0.99 × (height) − 1.49	166.4 (1)*	< 0.01
	M. togakushiensis	0.99				
height/length	≥ 40 mm					
	M. laevis	−1.24	86%	y = 36.42 × (height/length) − 18.55	1918.0 (1)**	< 0.01
	M. togakushiensis	0.97				
	< 40 mm					
	M. laevis	−1.17	86%	y = 44.86 × (height/length) − 24.48	420.9 (1)**	< 0.01
	M. togakushiensis	0.83				

* Those tested using MANOVA; ** those tested using one-way ANOVA

Table 3-4 Accuracy of linear discriminant function (LDF) for each region and size range

Predictor/Combination	Size class/Accuracy	
(a) length, height	40-100 mm	< 40 mm
All-East	93%	94%
All-West	70%	92%
East-East	92%	95%
West-West	75%	94%
(b) height/length	≥ 40 mm	< 40 mm
All-East	93%	86%
All-West	74%	86%
East-East	93%	94%
West-West	77%	84%

NOTE: In the Predictor/Combination column, the left-hand and right-hand side of the hyphen indicates the property of individuals used in constructing LDFs and those to which the LDFs were applied to test the accuracy, respectively. For example, "All-East" represents a combination in which the functions using all individuals collected in both regions was applied to the individuals collected in the east

Table 3-5 Linear discriminant function (LDF) constructed to identify two pearl mussels for each region and size range. Each alphabetical letter on the right side coincides with the accuracies in Table 3-6

Predictor/Region/Size class	Linear discriminant function	
(a) length, height		
East		
40-100 mm	$y = -0.29 \times (\text{length}) + 0.53 \times (\text{height}) + 1.55$	(a
< 40 mm	$y = -0.93 \times (\text{length}) + 1.80 \times (\text{height}) - 1.17$	(b
West		
40-100 mm	$y = -0.32 \times (\text{length}) + 0.68 \times (\text{height}) - 1.06$	(c
< 40 mm	$y = -1.31 \times (\text{length}) + 2.60 \times (\text{height}) - 2.29$	(d
(b) height/length		
East		
≥ 40 mm	$y = 38.75 \times (\text{height/length}) - 19.82$	(e
< 40 mm	$y = 49.92 \times (\text{height/length}) - 26.98$	(f
West		
≥ 40 mm	$y = 40.14 \times (\text{height/length}) - 19.90$	(g
< 40 mm	$y = 42.15 \times (\text{height/length}) - 23.10$	(h

Table 3-6 Accuracy of linear discriminant function (LDF) for each region and size range. Small alphabetical letters coincide with functions in Table 3-5

	Predictor/Region/Accuracy			
	length, height		height/length	
Size range (mm)	East	West	East	West
0-20	93%(b	96%(d	96%(f	88%(h
20-40	96%(b	92%(d	93%(f	79%(h
40-60	89%(a	85%(c	96%(e	79%(g
60-80	96%(a	67%(c	93%(e	67%(g
80-100	86%(a	73%(c	85%(e	80%(g

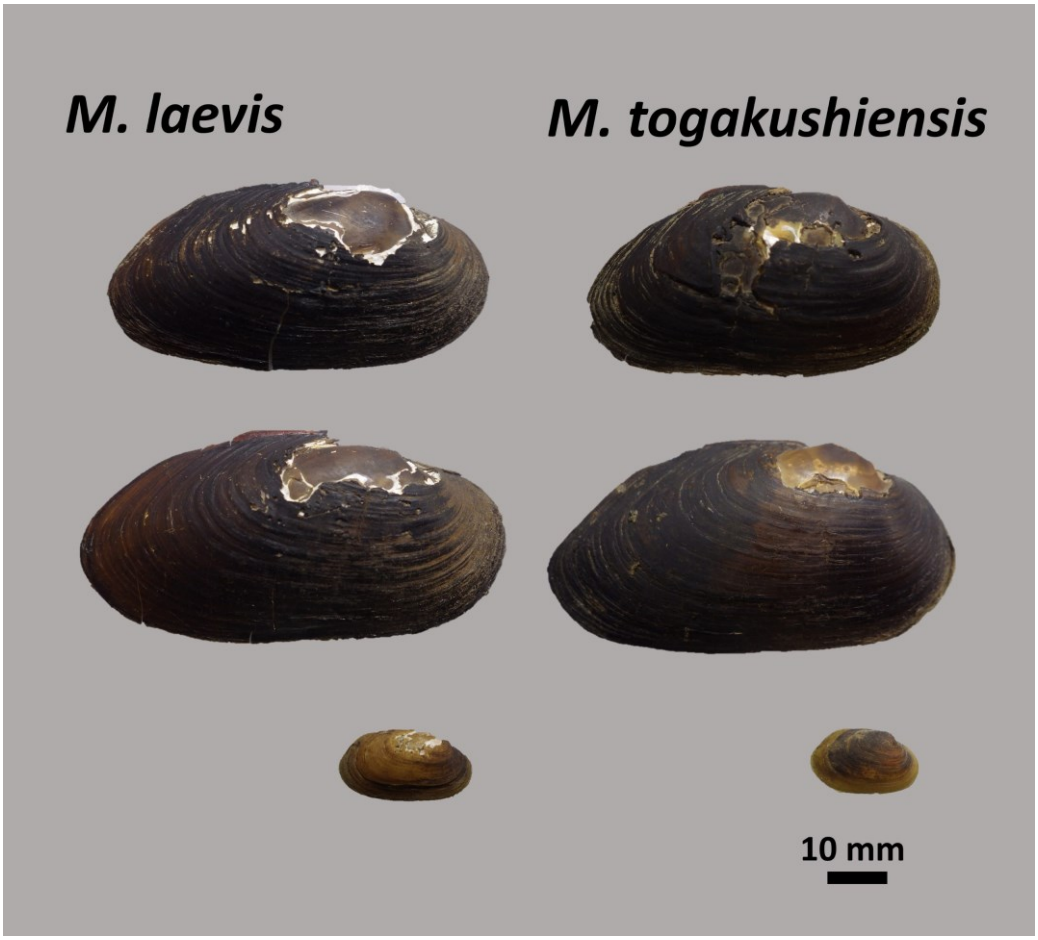


Fig. 3-1 Specimens of two pearl mussel species, *Margaritifera laevis* (left) and *M. togakushiensis* (right)

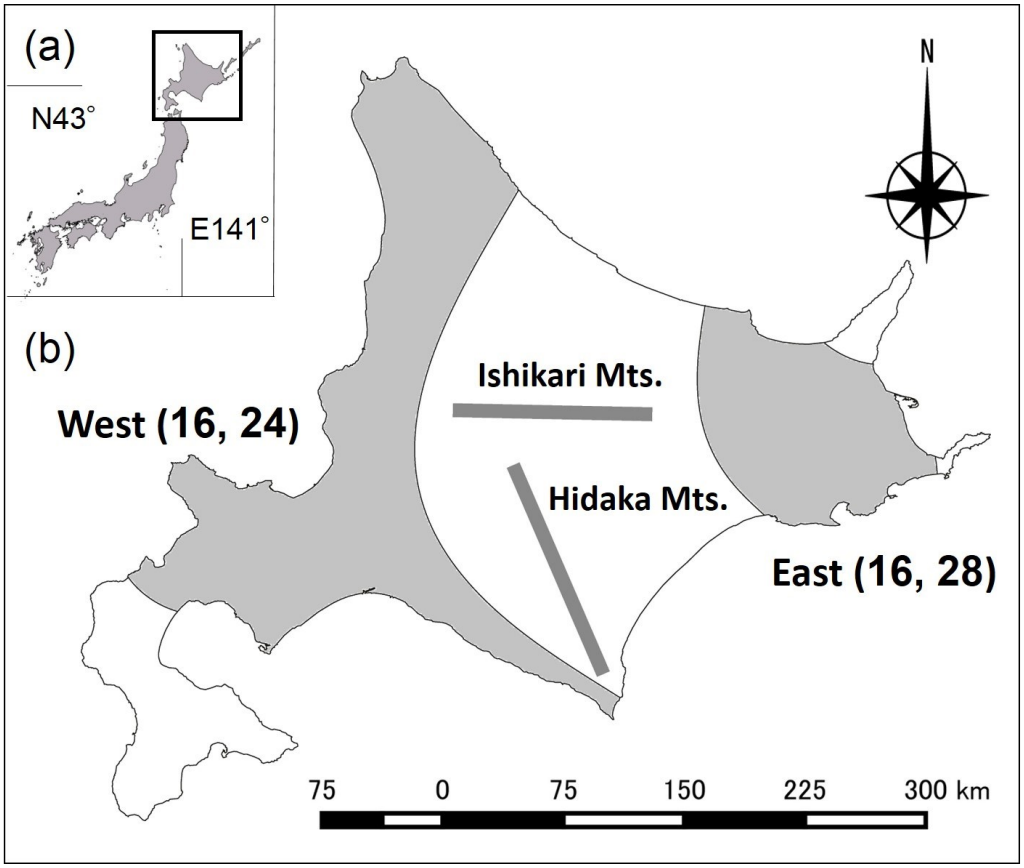


Fig. 3-2 Geographical location of Hokkaido (a) and the two study regions (east and west) (b). Numbers beside region name indicate the number of target watershed areas and rivers

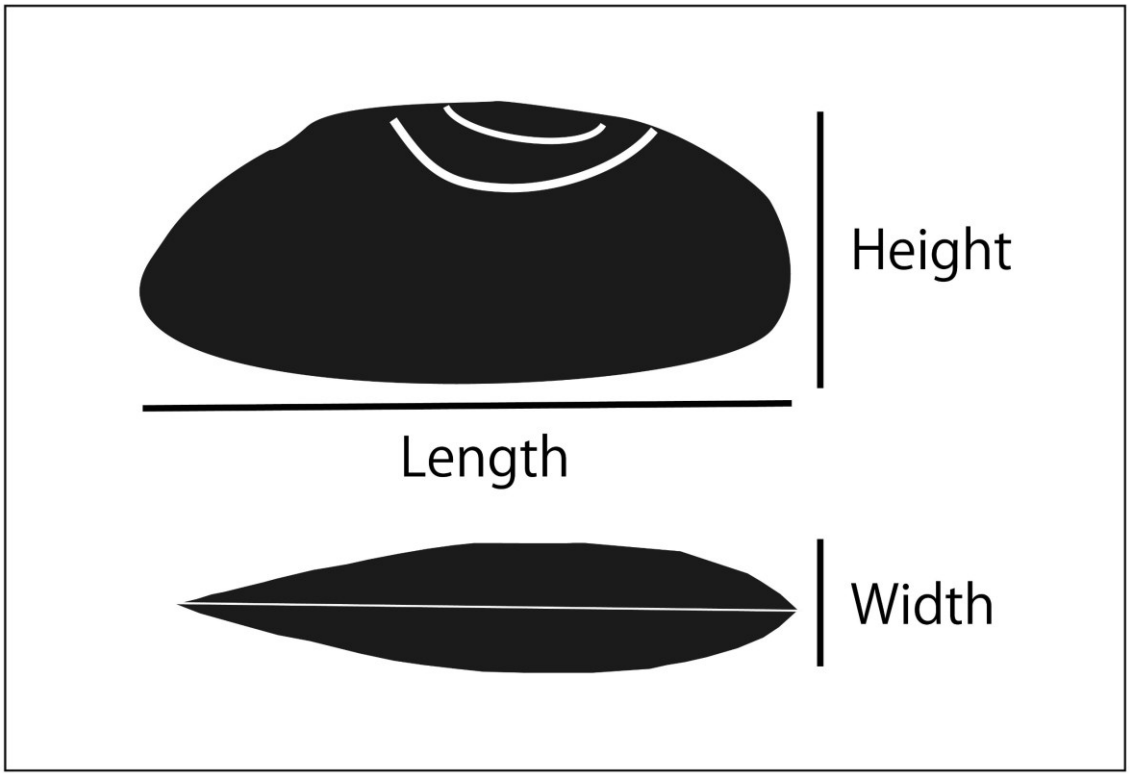


Fig. 3-3 Key morphological dimensions measured in the present study: Length, height, and width

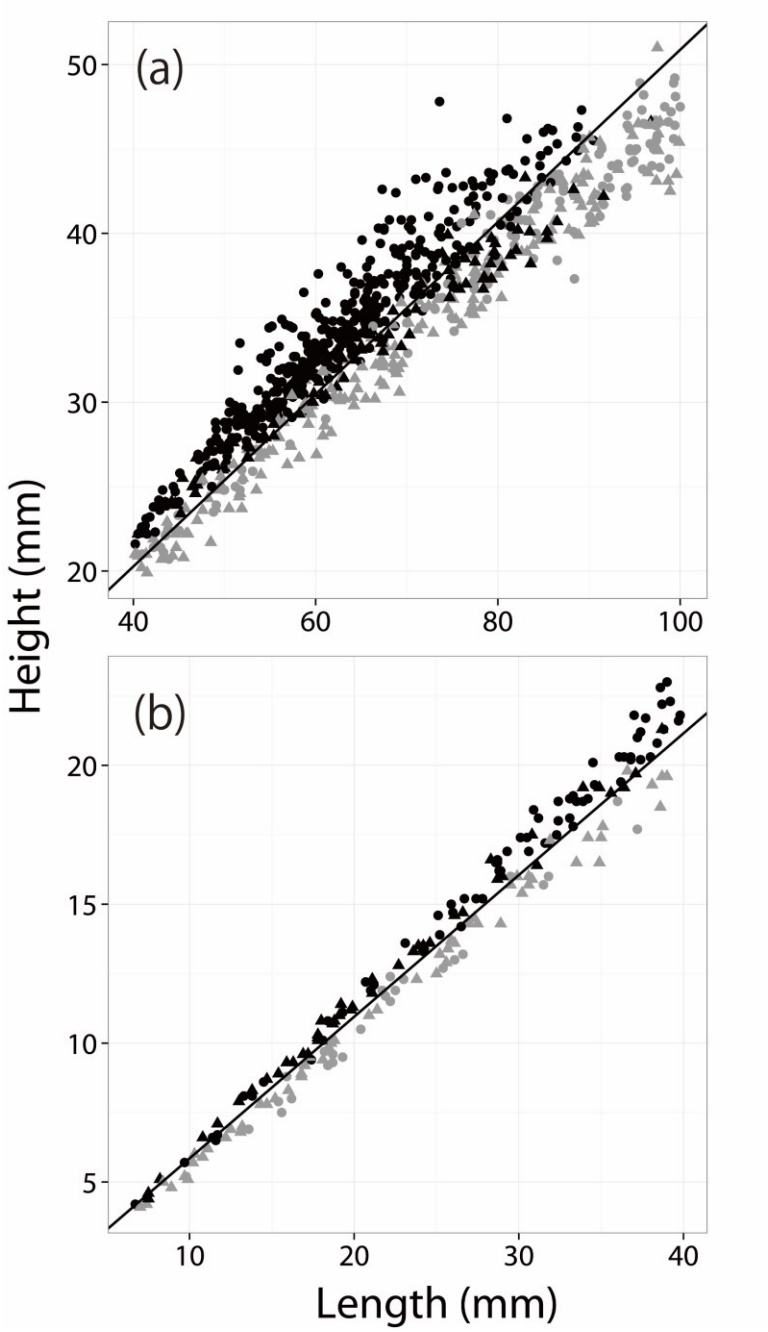


Fig. 3-4 Relationships between length and height of two pearl mussels in two size classes: 40–100 mm (a), and < 40 mm (b). Gray and black colors indicate *Margaritifera laevis* and *M. togakushiensis*, and circles and triangles represent east and west regions, respectively. Linear lines mean height = 0.51 × (length) − 0.13 (a) and height = 0.51 × (length) + 0.75 (b) lines solved from linear discriminant functions (LDFs)

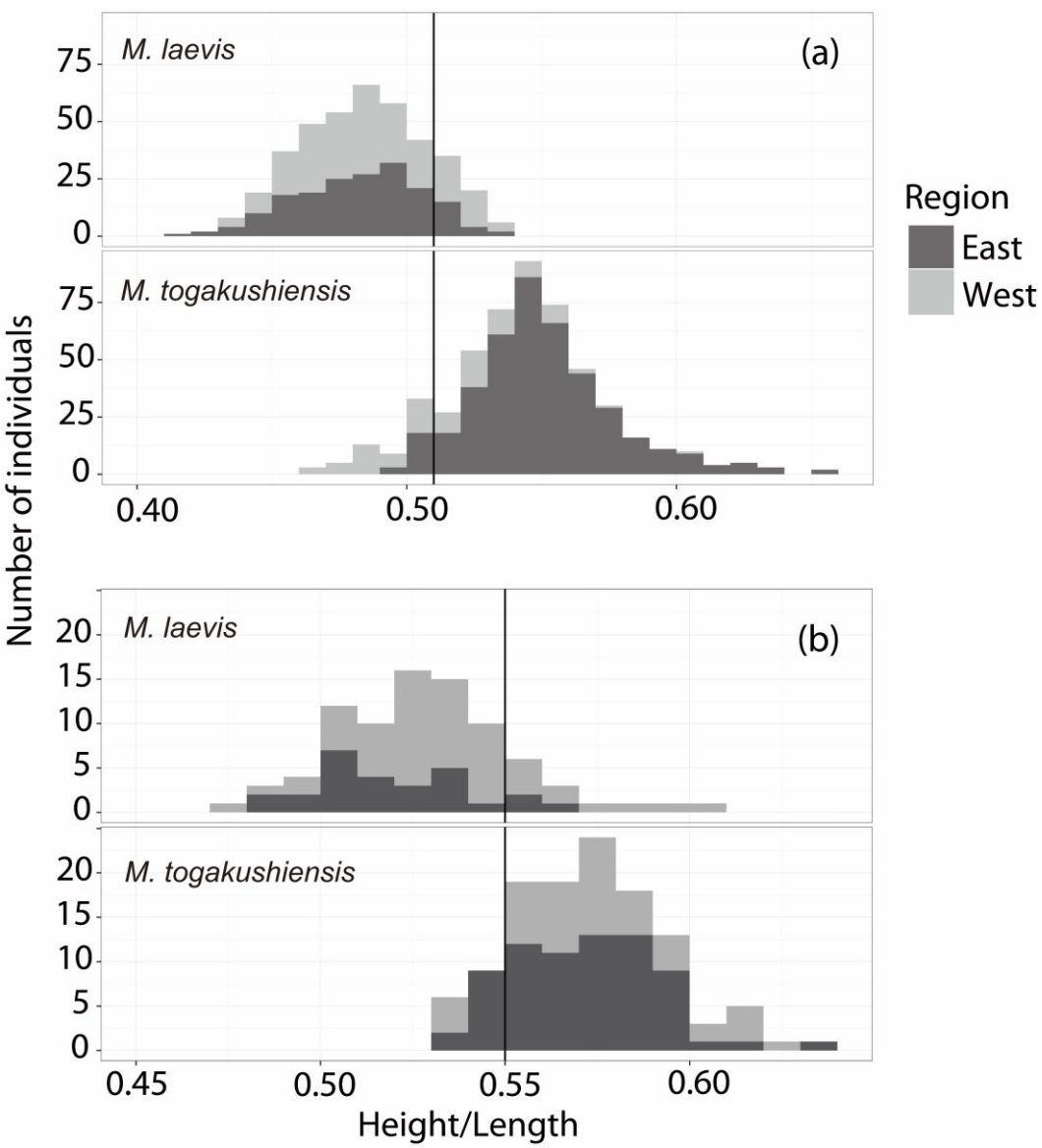


Fig. 3-5 Histograms of the height-to-length ratios for two pearl mussels in two size classes: ≥ 40 mm (a) and < 40 mm (b). Upper and lower stages indicate *Margaritifera laevis* and *M. togakushiensis*, and black and gray colors mean east and west regions, respectively. Linear lines mean height/length = 0.51 (a) and height/length = 0.55 (b) lines solved from linear discriminant functions (LDFs)

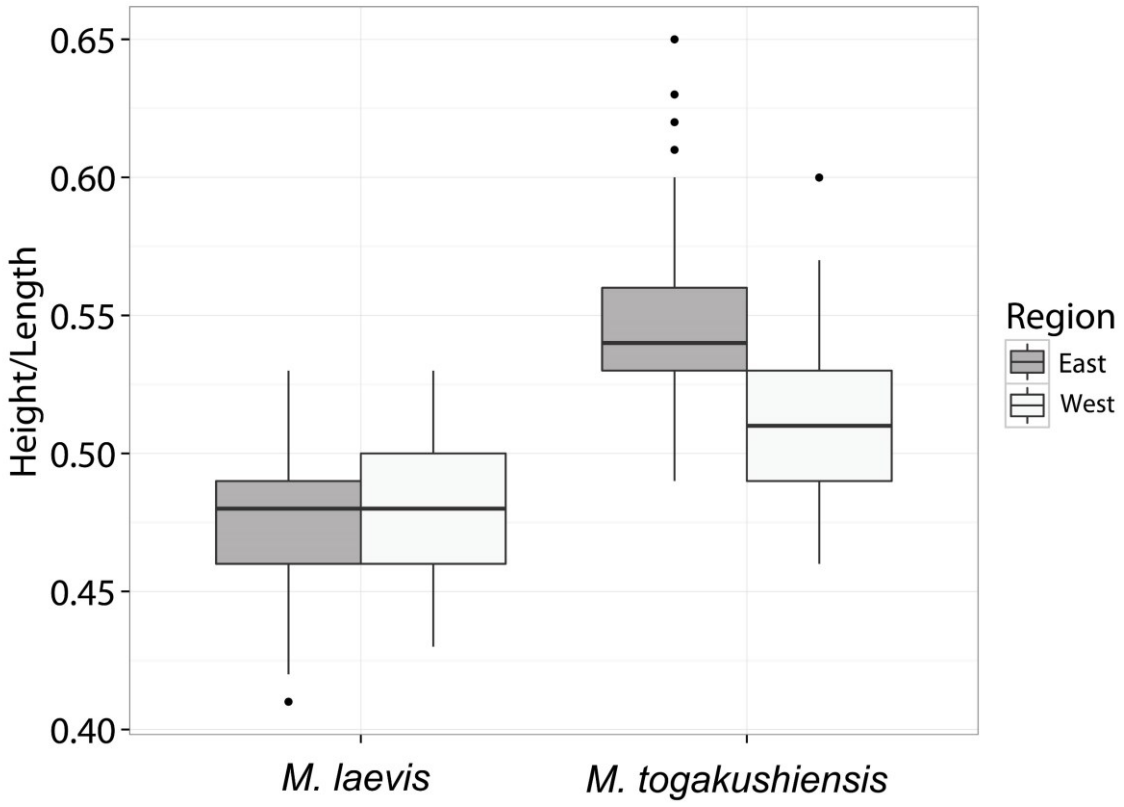


Fig. 3-6 Boxplots showing median (central thick lines), 25% and 75% quartile range around the median (box width) of height/length of ≥ 40mm individuals of two pearl mussels in each region

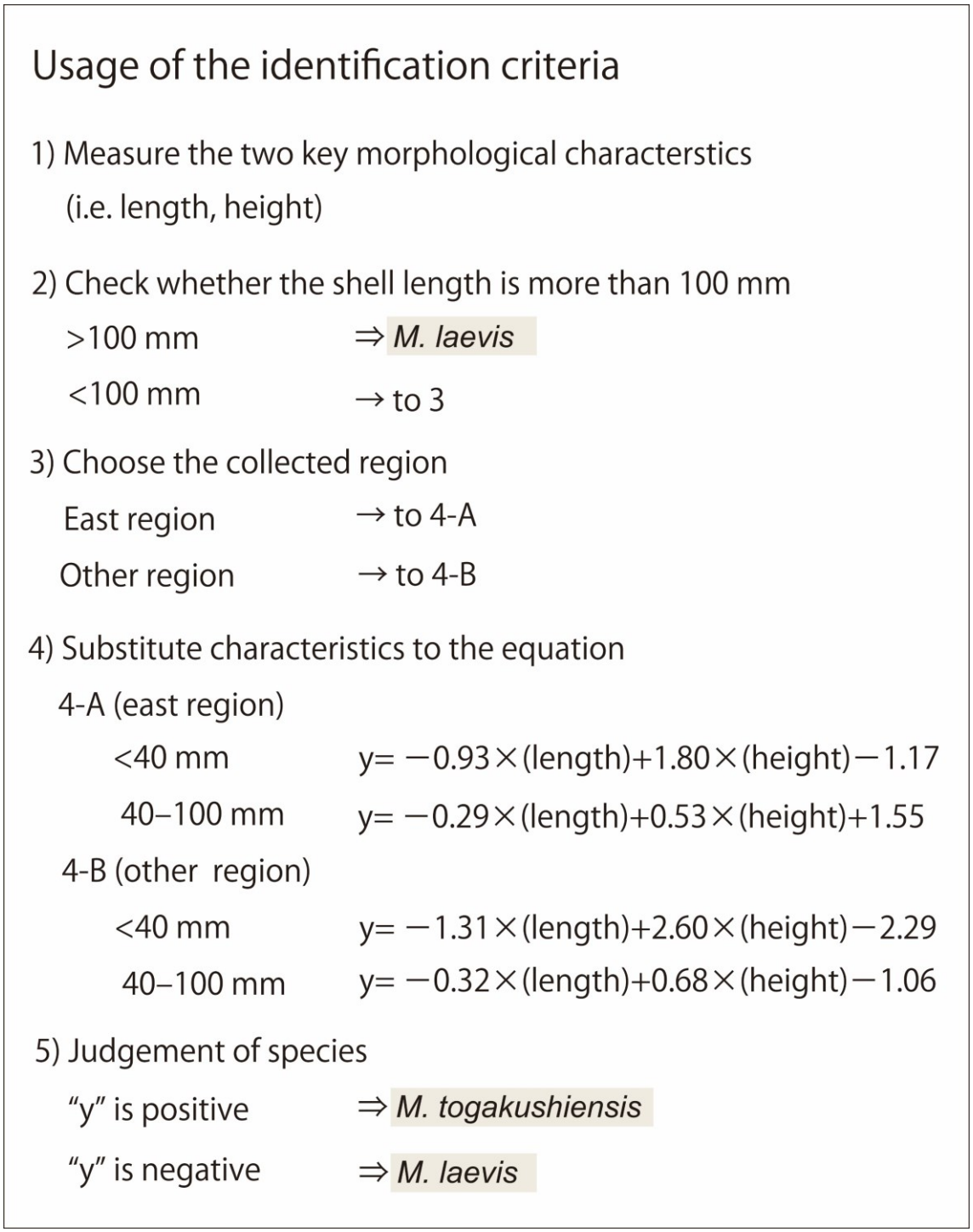


Fig. 3-7 Simple usage of the identification criteria of two pearl mussels

4. Population status of *Margaritifera togakushiensis* in eastern Hokkaido, northern Japan

4-1. Introduction

The decline of freshwater mussel populations has been confirmed worldwide in recent decades. Many researchers have sounded an alarm bell on the extinctions of freshwater mussel populations because of their important ecological functions such as water filtration, alterations of nutrient dynamics, creation of habitats for other organisms, and stabilizing and/or destabilizing (mixture) of bed-sediment (Vaughn and Hakenkamp 2001, Spooner and Vaughn 2006, Limm and Power 2011, Miura 2015, Vaughn and Hoellein 2018). The loss of the mussel population can cause irreversible changes in the structure and functions of freshwater ecosystems. Therefore, the conservation and management of freshwater mussels are important to maintain the functional integrity of freshwater ecosystems in the world.

One of the recent threats to freshwater mussel populations is recruitment failure. The many mussel populations have no signs of recent recruitment (presence of ≤ 10–20-years-old juveniles), which implies that extinction or declining of mussel abundance will happen in the future with a time-lag. Freshwater mussels are known as long-lived organisms in freshwater ecosystems (Haag and Rypel 2011). If recruitment once ceases, they can survive for a long time. There are also chances of recovery of natural recruitment. Therefore, screening of the non-recruiting populations is important for determining the population viability and prioritization of conservation targets (Boon et al. 2019). To do so, determining the status of mussel populations including age structure and signs of recent recruitment (i.e. presence/absence or the proportion of juveniles within a population) is necessary. However, except for a part of freshwater mussel species, the detailed population status including those is yet unknown.

Margaritifera togakushiensis is also recently threatened due to anthropogenic disturbances such as habitat fragmentation, river habitat modification, and land-use change (Ministry of the Environment of Japan 2018). However, the detailed population status of this species has not been determined yet. There are possibilities that even the remaining populations of *M. togakushiensis* have no signs of recent recruitment as with other unionida species. Compiling the information on the population status including age structure and recruitment is urgently needed to determine the

population viability of this species (Miura et al. 2019a), and its information can directly link to the prioritization of conservation targets of mussel populations (Geist 2010).

This chapter aimed to determine the population status of *M. togakushiensis* to compile basic information regarding population viability for the conservation of the target species. The status of age distribution and the proportion of juveniles of *M. togakushiensis* populations were especially focused on this chapter. Chapter 2 in this thesis showed that distributions of *M. togakushiensis* were relatively well confirmed in eastern Hokkaido (Fig. 2-5). Therefore, this chapter targeted on the streams in eastern Hokkaido. The ages of ≤ 20 years mussel individuals were defined as "juveniles" in the study in this chapter.

4-2. Methods

Study site

The study was conducted in 24 rivers across 8 watersheds in a total area of > 1500 km^2 of eastern Hokkaido, Japan, where several populations of *M. togakushiensis* were confirmed in Chapter 2 (Fig. 4-1, Table 4-1). The watersheds *A* to *G* and *H* run off into the Notsuke Strait and the Pacific Ocean. Each study river was not laid out on the upstream of another study river. The detailed locations of the study streams remain undisclosed owing to the conservation of the target species. Large dams and wires that hinder the migration of fish don't exist in all study rivers (Fukushima et al. 2007). *Noemachelius barbatulus toni*, *S. leucomaenis leucomaenis*, and *Pungitius* spp. are dominant fish species in the study streams (N. Ishiyama and K. Miura. unpublished data).

Population status

Field sampling to assess the population status of the target species was carried out from June to September 2017. Two ≥ 20-m study reaches [20.0–45.2 (mean 26.9 ± 22.83 SD) m length] were established on the glide conditions in each study river and were separated at least 500-m in watercourse distance from each other. Five transects were laid out at equal intervals in each study reach using a 50-m tape. In each transect, I placed three 0.09-m^2 quadrats, one at mid-channel and two at both sides separately, near the bank, for a total of 15 quadrats per reach. I collected mussels from each quadrat as follows. First, I collected all visible mussels using a glass-bottomed viewing

bucket. Second, I excavated the bed-sediment to a depth of ~10-cm using a trowel and immediately sieved all material through a 2-mm mesh sieve. This protocol can reduce the size bias (Hastie and Cosgrove 2002, Strayer and Malcom 2012). I collected at least 100 individuals of *Margaritifera* spp. from one study river to reach at least 50 individuals of *M. togakushiensis*. When the number of collected individuals of *Margaritifera* spp. by quadrat sampling was less than 100 individuals in a study river, I additionally collected the mussel individuals to be a total of 100 or more individuals. Then, one investigator found visually mussel individuals using a glass-bottomed viewing bucket by random walking in the study reach, and I laid out a quadrat with found individuals as the center and collected mussels in the same manner as above procedures. Collected individuals were photographed on the white tray with a ruler and a level gauge. Later, the shell length and height of each individual were measured using "Image J" software (https://imagej.nih.gov/ij/, 14 July 2019) as done in previous studies (Negishi et al. 2018).

Species identification

The species identification of two pearl mussel species was performed using the simple non-lethal identification criteria developed in Chapter 3. The criteria can accurately distinguish the two species using the linear discriminant functions (40–100 mm: $y = -0.29 \times (\text{length}) + 0.53 \times (\text{height}) + 1.55$, < 40 mm: $y = -0.93 \times (\text{length}) + 1.80 \times (\text{height}) - 1.17$) and the differences in the maximum shell length (> 100 mm is *M. laevis*) with high accuracies in the study region (86–96% with 92% mean). The detailed methods are shown in Figure 3-7. In addition, when linear discriminant functions were additionally applied to the individuals collected from study rivers and watersheds, those functions can more accurately distinguish the two species (94–100% with 97% mean). Thus, the constructed LDFs in Chapter 3 can identify the two pearl mussels with higher accuracies in the 24 study rivers in this chapter.

Age determination

I developed the growth models of *M. togakushiensis* to determine the age structure and identify the ages of ≤ 20 years individuals. The age of margaritiferids can be estimated by the

relationship between shell length and the number of growth rings on the ligament (e.g. Hastie et al. 2000b). I collected 23–83 live individuals of *M. togakushiensis* (mean: 42 individuals/watershed) from each watershed from 2017 to 2018 (Table 4-1). The collected individuals were identified using the above criteria and/or the presence of matured glochidium larvae in the gills of female individuals on the occasion of the reproductive season (Kondo 2008, Miura et al. 2019b). After the shell and ligament length of each individual were measured, the number of growth rings was counted under a stereomicroscope. A part of the ligaments of margaritiferids is usually abraded due to the erosion. Thus, the number of growth rings on the abraded part of the shell was estimated on the basis of the relationship between shell length and the number of growth rings obtained from 28 non-eroded juvenile shells (length: 7.6–22.0 mm) done as with Hendelberg (1961). I fitted four non-linear models: Bertalanffy growth function, Hyperbolic saturation function, Logistic function, Gompertz function using measured shell length and estimated age of each individual. These models have been traditionally used to describe the growth characteristics for freshwater and marine bivalves including margaritiferids (San Miguel et al. 2004, Akiyama and Iwakuma 2010, Haag and Rypel 2011, Nakamura et al. 2018). The four models are expressed as the following functions.

Bertalanffy growth function

$$L_t = L_\infty(1 - e^{-k(t-t_0)})$$

Hyperbolic saturation function

$$L_t = \frac{L_\infty k(t - t_0)}{1 + k(t - t_0)}$$

Logistic function

$$L_t = \frac{L_\infty}{1 + e^{-k(t-b)}}$$

Gompertz function

$$L_t = L_\infty e^{-ae^{-kt}}$$

where L_∞ is the theoretical maximum length (or asymptotic length, mm), k is the growth coefficient (year^{-1}), t_0 is the theoretical age at zero-length (year), and a and b (year) are constants The best-

fitting model was selected by comparing RSS (residual sum of squares) (Akiyama and Iwakuma 2010). After the construction of the best-fitted model of each watershed, the age of each individual was estimated using shell length measured from the images taken in the field sampling. The histogram of the age composition of each population was charted combining the data of two reach in each study river.

Estimation of the population viability

Population viabilities of freshwater mussels have been assessed by the presence/absence or the proportion of ≤ 10–20-years-old juveniles within the population (Cosgrove et al. 2000, Geist 2010, Österling et al. 2010). According to a recent-developed standard approach for monitoring of freshwater pearl mussel populations, at least 20% of the population should be ≤ 20-years-old individuals to determine whether the population is recruiting adequately (Hastie and Cosgrove 2002, Boon et al. 2019). Therefore, the populations having at least 20% of ≤ 20-years-old individuals were considered to be "functional" populations in this chapter.

4-3. Results

A total of 5078 individuals of *Margaritifera* spp. was collected from the 24 rivers across the eight watersheds in the study region. 2491 individuals were identified as *M. togakushiensis* by the identification criteria of the two species. The numbers of identified *M. togakushiensis* individuals in each river were more than 50 individuals (N = 55–211) whereas less than 50 individuals were in four rivers (Rivers *l*, *m*, *r*, and *s*, N = 14–47) (Table 4-1). As a result of the comparisons of fitting curves of age estimation functions, Hyperbolic saturation function and Gompertz function were the most fitted function in seven study watersheds (*A*, *B*, *C*, *D*, *E*, *G*, and *H*) and one watershed (*F*) respectively, that is supported by the lowest RSS of the comparison of four functions in each watershed (Table 4-2). The shell length of individuals estimated as 20-years in each watershed was 37.9–54.2 mm calculated from the length-age relationship (Table 4-1). From the estimated age distribution of each river, there was a gradient in the proportion of juveniles (0.00–0.54) among the 24 study rivers, and juveniles were not found from four study rivers (Rivers *p*, *q*, *s*, and *w*) (Fig. 4-2, Table 4-1). A total of seven populations were estimated as "functional" populations among the

24 populations. The maximum ages of individuals were more than 100 years in 10 of 24 study rivers (Table 4-1), whereas the youngest maximum age was 39.5 years in River *l*. The maximum ages ranged from 57.4 to > 100 years among the four non-juvenile study rivers. The minimum ages of *M. togakushiensis* individuals were 24.3–28.4 years in the four non-juvenile study rivers.

4-4. Discussion

The study in this chapter provides a first report of the population status of *M. togakushiensis* mainly focusing on recruitment, which is one of the indicators of population viability. As with the recent global trends of the population status of freshwater mussels (Bauer 1988, Outeiro et al. 2008, Larsen 2010, Sousa et al. 2013, Lois et al. 2014), at least four *M. togakushiensis* populations had no signs of recent recruitment among 24 populations. In addition, 17 populations that had lower than 20% proportion of juveniles were considered as non-functional populations among 24 populations in eastern Hokkaido. There was a gradient (0.02–0.54) in the proportion of juveniles among the with-juvenile populations. The maximum proportion of the juveniles among 24 rivers reached up to 0.54 in River *x*. The results suggested that the highest priority should be given to the preservation of the seven functional populations for the conservation of *M. togakushiensis* in the study region. In addition, among 17 non-functional populations, four populations are seriously threatened by recruitment failure. When trying to restore natural recruitment, in particular, these four non-recruiting populations should be paid attention among 17 non-functional populations for the conservation of target species in the study region.

Based on the classification by Boon et al. (2019), seven populations (29.1%) with recent recruitment can be regarded as viable populations in the study region. Geist (2010) reviewed the studies investigating the status of *M. margaritifera* from 2003 to 2009 in Europe, which showed that the spatial distribution of known populations and its status of recruitment (with or without > 20% of ≤ 20-years-old juveniles) on the map. Although Geist (2010) doesn't show the total number of marks of the distribution on the map, 26.1 % (30 of 115 individuals) of populations were estimated as "functional" population when I counted the number of visible marks (Fig. 1 in Geist (2010)). If I compare the present results with the reports in Geist (2010), the ratios of functional populations are comparable (29.1% vs. 26.1%). In addition, Akiyama (2007) investigated the current status of 12

populations of *M. laevis* in the sites from Hokkaido and Honshu, Japan. All *M. laevis* populations contained more than 20% of ≤ 20-year-old juveniles. These imply that *M. togakushiensis* populations in my study regions are threatened by recruitment failure to the extent similar to those of *M. margaritifera* in Europe, and more threatened compared with *M. laevis* in Japan. However, the geographical distribution range of *M. togakushiensis* is narrower and the number of known populations is less than above two margaritiferids (Kondo 2008, Geist 2010, Lopes-Lima et al. 2018). Thus, greater attention should be paid to the risks of extinction and/or decline of *M. togakushiensis* populations in Japan.

The predominance of large (old) individuals and lack of small (young) individuals of freshwater mussels have been commonly reported in the previous studies (Österling et al. 2010, Strayer and Malcom 2012, Reid et al. 2013). My results also dominantly contained this type of age structure (i.e. age distribution of less than 20% comprising of ≤ 20-year-old individuals). This pattern could be caused by the following two reasons. First, recent recruitment is impaired by the consequences of human activities such as habitat degradation and habitat fragmentation (Awakura 1969, Österling et al. 2010, Strayer and Malcom 2012). The reduced recruitments of juveniles can lead to the absence of young age individuals in the populations. Second, the mussel sampling can be biased such that small-sized individuals, which are difficult to detect by visual methods, are undercounted (Hastie and Cosgrove 2002). Therefore, many studies have used methods with excavating and sieving the river-bed materials to reduce sampling bias (Österling et al. 2010, Strayer and Malcom 2012, Terui et al. 2014b). Most studies targeting the healthy mussel populations in unpolluted and unfragmented streams with unbiased sampling have shown strongly right-skewed age distribution dominated by young age individuals (Haag 2012). In the study in this chapter, I used unbiased methods and showed that the age structure of the populations having high proportions of the juveniles (0.52 and 0.54 in Rivers *a* and *x*) formed right-skewed distributions (Fig. 4-2). These indicate that my methods could detect young individuals properly. Therefore, I deny the second reason and infer that the lack of young age individuals in several populations in my study is caused by human impacts.

Twenty-four to twenty-eight years have already passed since the recruitment cessation of four non-juvenile populations, which were estimated by age estimations. When the years are

subtracted from the survey year (2017), recruitment has been stopped since 1989–1993. The landscape in the study region is known to be one of the most agriculture-dominated in Hokkaido. The development of the vast agricultural lands (mainly pastureland and meadow) begun in the 1950s and 90% of current agricultural lands were developed by the late 1980s. In addition, the number of cattle peaked in the 2000s, and nutrient inputs to the rivers from agricultural land dramatically increased in response to these changes in land-use and the intensity of agriculture (Nagasaka 2017). The timing of the recruitment cessation of *M. togakushiensis* in four non-juvenile populations well coincides with the period of these changes. The habitat deterioration relating development of agricultural lands such as excessive fine sediment load and nutrient inputs can cause the recruitment failure of freshwater mussel populations by decreasing juvenile survival and/or reducing the host fish availability (Österling et al. 2010, Strayer and Malcom 2012, Österling 2019). Therefore, recruitment failure of *M. togakushiensis* in my study region might be also influenced by habitat deterioration caused by land-use changes to agricultural lands in recent decades.

The study in this chapter is the first case attempting the age estimation of *M. togakushiensis*. The age estimation revealed that the maximum age of *M. togakushiensis* is more than 100 years in the study region. However, the maximum ages of *M. togakushiensis* differed among 24 populations. This result could provide important information for prioritizing conservation targets. For example, among four non-recruiting rivers (i.e. Rivers *p*, *q*, *s*, and *w*) in which there were no signs of recent recruitment, the estimated remaining times till extinction of Rivers *p* and *w* were 74.0 and 71.7 years respectively when assuming the maximum age is 100 years. On the other hand, those of Rivers *q* and *s* were 48.1 and 31.0 years, and there was a maximum difference age was 43.1 years among the four rivers. It means that populations in Rivers *q* and *s* could become extinct earlier than those in Rivers *p* and *w*. If the recovery of natural recruitments of the four *M. togakushiensis* populations is considered, Rivers *q* and *s* only have less than 30–40 years before the projected local extinctions while Rivers *p* and *w* are provided with at least 70 years.

The study in this chapter clarified the current population status of *M. togakushiensis*, especially regarding recruitment, in eastern Hokkaido. A total of seven populations were adequately recruiting. However, recruitment of a total of 17 populations may be suppressed by

land-use changes and associated human activities. To prevent the extinction and dramatic decline of *M. togakushiensis*, the determining of the causes of recruitment failure is urgently required. However, the mechanisms of recruitment failure of freshwater mussels are poorly understood in the world (Haag et al. 2019). The identification of the causes of recruitment failure of *M. togakushiensis* populations is critically important to prevent the extinction of *M togakushiensis*. Bridging the research gap is the purpose of the next chapter. The further studies and conservation of *M. togakushiensis* using the present results would lead to maintaining the healthy populations, and consequently help to maintain important ecosystem functions of *M. togakushiensis* in eastern Hokkaido.

Table 4-1 The summary of information on the population status of *M. togakushiensis* in 24 study rivers

Watershed	River	N	Prop. of juv. inds.*	Status of viability**	Max. age (year)	Min. age (year)
A	*a*	129	0.52	functional	> 100	8.49
	b	108	0.18	no	> 100	8.56
	c	57	0.26	functional	> 100	13.14
B	*d*	171	0.16	no	68.53	0.12
C	*e*	211	0.03	no	63.22	11.15
D	*f*	114	0.03	no	> 100	10.76
	g	122	0.02	no	92.55	7.62
	h	116	0.06	no	90.83	14.96
E	*i*	129	0.20	functional	96.17	7.34
	j	75	0.17	no	92.87	15.53
F	*k*	69	0.03	no	> 100	13.07
	l	14	0.43	functional	39.52	2.00
G	*m*	39	0.26	functional	85.98	12.93
	n	63	0.22	functional	> 100	7.84
	o	55	0.02	no	> 100	11.79
H	*p*	92	0.00	no	> 100	25.98
	q	111	0.00	no	72.46	24.32
	r	47	0.09	no	51.10	9.59
	s	44	0.00	no	57.36	26.39
	t	149	0.10	no	64.43	9.01
	u	153	0.10	no	79.03	3.42
	v	189	0.12	no	80.85	4.13
	w	104	0.00	no	> 100	28.35
	x	130	0.54	functional	> 100	3.99

*Proportion of juvenile individuals: the proportion of the ≤ 20-years-old juveniles in the population

**Status of viability: the populations having at least 20% of ≤ 20-years-old individuals were considered to be "functional"

Table 4-2 The summary of the results of fitting four non-linear models to each *M. togakushiensis* individuals

Watershed	Most fitted function	RSS*	N**	Length (mm) of 20 yr individuals
A	Hyperbolic	809.90	48	54.20
B	Hyperbolic	637.00	30	39.40
C	Hyperbolic	215.40	23	41.50
D	Hyperbolic	1072.00	42	37.90
E	Hyperbolic	745.10	46	47.30
F	Gompertz	306.60	29	39.00
G	Hyperbolic	856.10	31	41.60
H	Hyperbolic	1913.00	83	44.50

*RSS: residual sum of squares

**N: Number of samples

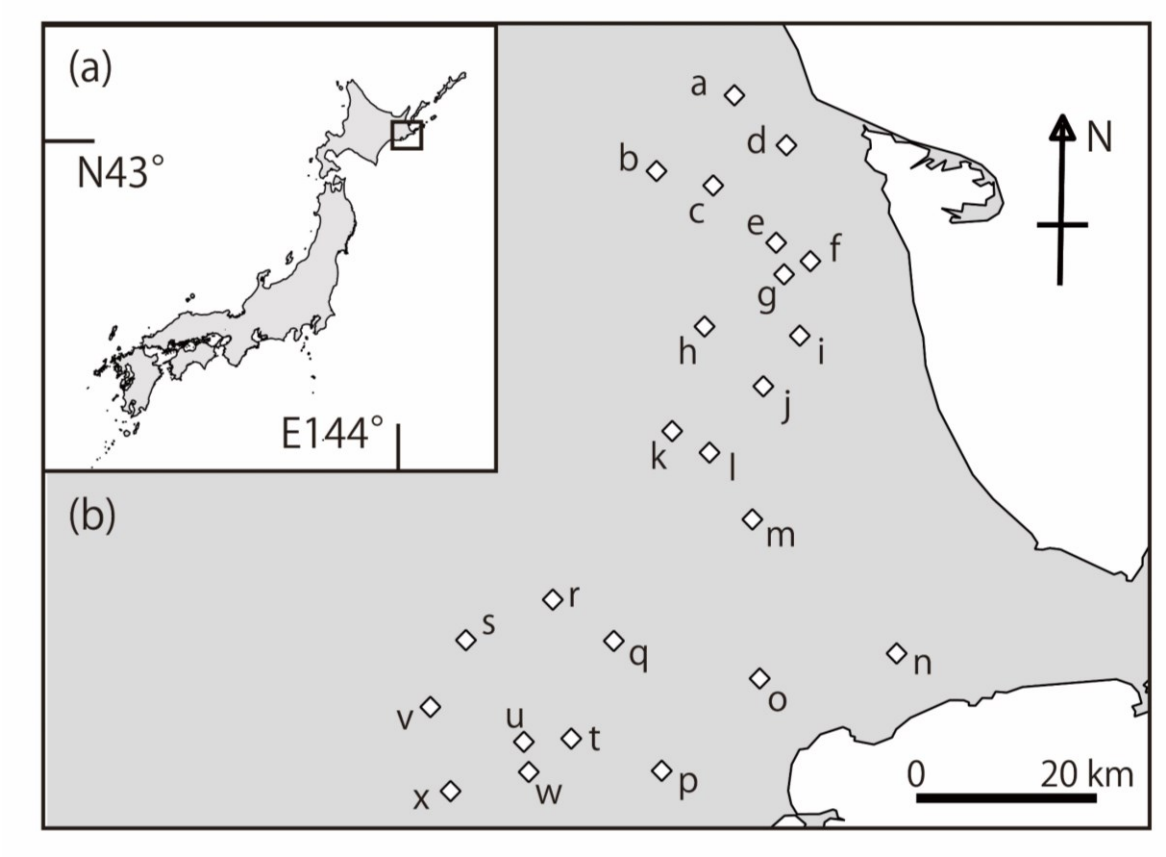


Fig. 4-1 The geographical location of the study region (a) and the study rivers (b). White diamonds indicate the location of the study reach. Alphabets mean the name of each study river

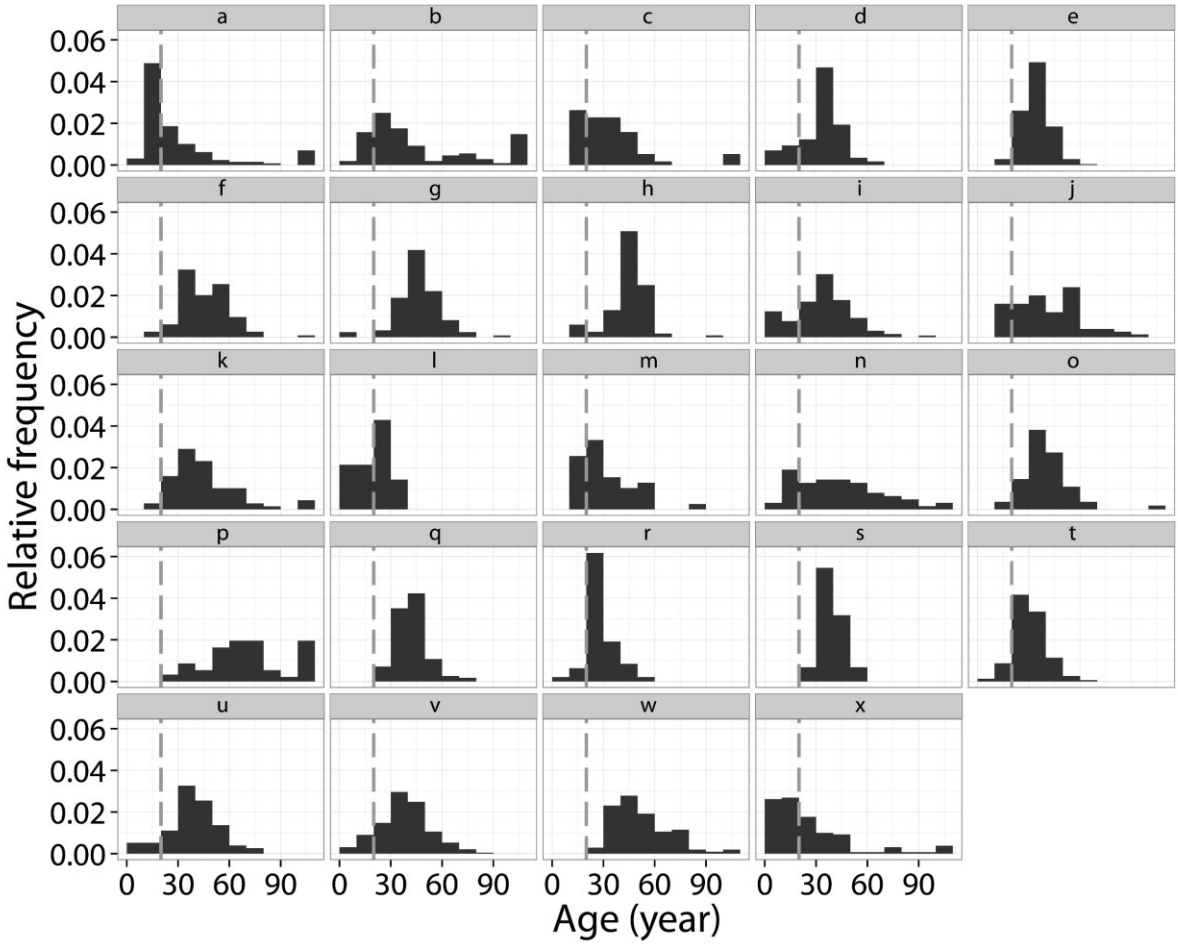


Fig. 4-2 The relative frequency distributions of the age of *M. togakushiensis* populations in 24 study rivers. Gray dashed lines indicate the age of 20-year-old juveniles

5. Processes of recruitment failure of long-lived endangered freshwater pearl mussel (*Margaritifera togakushiensis*)

5-1. Introduction

Human activities have caused population declines and species extinction of wildlife worldwide. The recent loss of biodiversity is often called as sixth mass extinction. Determining the cause of extinction is one of the most important issues in conservation biology (Wake and Vredenburg 2008, Barnosky et al. 2011, Ceballos et al. 2015).

Recent studies have pointed out the threats of delayed extinction in various ecosystems (so-called "extinction debt", Kuussaari et al. 2009, Dullinger et al. 2013, Halley et al. 2016). Local populations and species can become extinct with a considerable long time (several months to several thousands of years) after any disturbances and perturbations (Figueiredo et al. 2019). This happens because the species with long generation times and populations near their extinction threshold can persist for a long time even the habitat condition is unpreferable. On the other hand, as long as species or populations that are predicted to become extinct still persist, there is time for conservation measures such as habitat restoration and landscape management by reaching a new equilibrium (the state that the number of species or abundance does not change). However, only few cases have succeeded in the prevention of the time-lagged extinction (Kuussaari et al. 2009). One of the reasons for the lack of cases that prevented time-lagged extinction is a poor understanding of the mechanisms of extinction debt.

The previous studies have demonstrated that habitat loss and fragmentation are the main causes of extinction debt (e.g. Helm et al. 2006, Mouquet et al. 2011). Both can decrease population viability by the reduced habitat area and increased isolation but species or individuals can persist for a long time in the small and isolated habitats (i.e. "metapopulation theory", Hanski and Ovaskainen 2002). Recent studies have also shown that habitat deterioration, which is one of the major reasons of extinction (Wilcove et al. 1998, Dudgeon et al. 2006, Reid et al. 2018), can also cause the extinction debt (Gonzalez-Varo et al. 2015, Sand-Jensen et al. 2017). However, there is a lack of information on the process of the extinction debt caused by habitat deterioration. This situation can make it difficult for the effective conservation of the target species to be implemented

even though signs of extinction debt can be recognized. Therefore, the empirical understanding of the detailed process of extinction debt caused by habitat deterioration is urgently required.

Freshwater mussels have been subjected to high rates of extinction or decline in recent decades (Ricciardi and Rasmussen 1999, Lydeard et al. 2004). Many populations are facing a high risk of extirpation due to recruitment failure (many populations, although containing adults, completely lack juveniles; Österling et al. 2008, Strayer and Malcom 2012, Reid et al. 2013). Recruitment failure of freshwater mussels including *M. togakushiensis* can be considered to be an example of extinction debt because adult individuals can survive for a long time after recruitment ceased (Haag 2012, Ferreira-Rodríguez et al. 2019). Recruitment cessation of long-lived freshwater mussel species is potentially usable as models to show the detailed process of the extinction debt (Hylander and Ehrlén 2013). The determination of the detailed processes of recruitment failure of freshwater mussels may lead us to a better understanding of the reasons why the extinction debt of long-lived species occurs. In addition, such information is necessary to prevent the extinction of freshwater mussel populations globally.

Previous studies attempted to determine the causes of recruitment failure of freshwater mussels, and provided suggestive results about the negative effects of fragmentation, habitat deterioration, and decline or disappearance of suitable host fish (Österling et al. 2008, 2010, Strayer and Malcom 2012). However, freshwater mussels have complex life histories including the parasitic phase in their larval stage (Fig. 1-1). A comprehensive test of which life history regulates recruitment is necessary to appreciate the mechanisms and drivers by which the recruitment failure of freshwater mussels is caused. However, most previous studies have focused only on a part of their life-history stages (Geist and Auerswald 2007, Österling et al. 2008, 2010, Strayer and Malcom 2012, Haag and Stoeckel 2015, Galbraith et al. 2018, Haag et al. 2019). This knowledge is not always sufficient in attempts to effectively recover the natural recruitment of freshwater mussels in the actual conservation measures because impaired recruitment might have been caused by problems in multiple stages of life histories (e.g. Galbraith et al. 2018).

The aim of Chapter 5 was to reveal the processes of the recruitment failure of *M. togakushiensis*. The content is composed of two parts. First, I explained the general life histories of freshwater mussels including *M. togakushiensis* and how to demonstrate the process of recruitment

failure of the target species in a theoretical framework. Second, I conducted field observations and experiments to test the relationships among recruitment, life-history stage indices indicating reproductive efficiency, and environmental factors in the 11 study rivers chosen from the 24 rivers targeted in Chapter 4.

5-2. A theoretical framework

This section summarizes the general life histories of freshwater mussels with an explanation of the processes of the reproduction and related important indices of mussels focused in this chapter. In addition, biotic and abiotic factors potentially regulating recruitment efficiency are introduced in relation to how to determine the process of recruitment failure.

Adult stage

Freshwater mussels are typically sedentary and they bury their part or most of their body in the adult stage (Fig. 1-1). Freshwater mussels are generally hermaphrodite. Matured females inhale the sperm sphere released from matured males through inhalant siphons in their reproductive period, and they fertilize to produce glochidium larvae in the gills (Haag 2012). Matured glochidia are finally released to the water column through exhalant siphons of females. Therefore, the potential amount of larval production of the population is mainly determined by the abundance of matured gravid individuals. Sufficient amount of larval production is necessary for higher recruitment efficiency (McLain and Ross 2005). Gravidity rate (the proportion of the gravid individuals within the population) has been widely used as the indicator of fertilization success and producing matured glochidium larvae in the previous studies (Moles and Layzer 2008, Österling et al. 2008). Gravid individuals can be defined by the presence/absence of matured glochidium larvae in the outer demibranchs of adult individuals (e.g. Hastie and Young 2003, Negishi et al. 2018). The gravid abundance of the population can be described as the product of the gravidity rate and adult mussel density; this was defined as "gravid density" and used as an indicator of the potential amount of larval production in this study (Fig. 5-1). Therefore, gravid density is a function of gravidity rate and thus is strongly affected by gravidity rate. There are three major extrinsic factors that are known to affect gravidity rate and adult mussel density. Increases

in the total suspended solids (TSS) in the water column can inhibit fertilization success and glochidia production (Gascho Landis et al. 2013, Gascho Landis and Stoeckel 2016). Water temperature is also an important factor for the maturity of glochidia (Hastie and Young 2003). Nutrient concentrations have been also suspected as an influential factor for fertilization success and glochidia production (Bauer 1988). In addition to them, physical environmental factors (e.g. depth and velocity) can also affect both of gravidity rate and adult density.

Parasitic stage

Released glochidia from female mussels are viable for about 24 h in the water column. They attach the host tissues when encountering the host fish individuals, and the mussels can proceed to the parasitic stage (Fig. 5-1, Haag 2012). Attached glochidia are completely encysted by host tissues within several days. However, larvae are perished within up to two weeks if they cannot tolerate the immunity of host fish (Ziuganov et al. 1994). Therefore, the potential number of successful juvenile supplies to the population is mainly determined after this initial period of reduction in abundance from immune responses ceased (Fig. 5-1). The infection success of glochidia can be judged by observation of the host tissues after two weeks from the attachment (Österling et al. 2008). Encysted glochidia live on the host fish from several days to several months (Strayer 2008). After metamorphose, juveniles detach themselves from host fish and reach to the riverbed. Sufficient number of juvenile supplies to the population is necessary for higher recruitment efficiency (Österling et al. 2008). The potential number of juvenile supplies can be described as a product of host fish density and the mean number of glochidia load on a host fish, and is referred to as "glochidia density" (Österling et al. 2008, Schneider et al. 2019). The study used glochidia density as an indicator of the potential number of juveniles (Fig. 5-1b). The previous study showed that the mean glochidia load on a host fish has a positive relationship with mussel density (Österling et al. 2008). It may be because the higher potential amount of larval production can contribute to higher concentrations of glochidia in the water column, and eventually increase the probability of the encounter of glochidia to host fish glochidia and subsequent infection. Glochidia density could potentially have positive relationships with the density of gravid mussels. Host fish density, and glochidia attachment and metamorphosis can be influenced by habitat

deterioration represented by increasing nutrient concentration (e.g. $N\text{-}NO_3^-$) and total suspended solids (Beussink 2007, Moore and Bringolf 2018, Österling 2019). In addition, water temperature potentially affects infection success (Akiyama and Iwakuma 2007, Taeubert et al. 2014).

Post-parasitic juvenile stage

Metamorphosed and detached juveniles fall to the riverbed and proceed to the post-parasitic juvenile stage (Fig. 5-1). Post-parasitic juveniles live in the sediments of the riverbed from several years to decades (Haag 2012). The survived juveniles in this life-history stage can proceed to the adult stage after maturity. Therefore, the survival rate of juveniles may positively affect the recruitment of freshwater mussels. This life-history stage is generally considered as the most sensitive life-history stage to habitat deterioration. In particular, just metamorphosed and detached juveniles may be the most sensitive stage in the post-parasitic juvenile stage (Buddensiek 1995). Several previous studies showed that the proportion of juveniles (or juvenile density), which is an alternative indicator of juvenile survival, was negatively affected by excessive fine sediment (both organic and inorganic particles) and high nutrient concentration, suggesting that juvenile survival can be reduced by those two factors (Österling et al. 2010, Strayer and Malcom 2012). In addition, dissolved oxygen and pH, and water temperature can also affect juvenile survival (Buddensiek 1995, Geist and Auerswald 2007, Wang et al. 2008).

An approach in empirical testing

As stated above, recruitment of freshwater mussels can be stopped or slowed (i.e. decreasing the proportion of the juveniles or absence of juveniles) when any stages of their life history are disrupted. Therefore, identification of key life-history stage(s) regulating recruitment is possible by testing the relationship between the degree of successful recruitment and indicator of each life-history stage (i.e. gravid density, glochidia density, and juvenile survival rate). Therefore, this study first tested the relationship between each life-history stage indicator and the proportion of juveniles within the population. Second, abiotic and biotic factors limiting the key indicator of the life-history stage were explored. A prediction was that indicators limiting recruitment efficiency can have a positive relationship with the proportion of juveniles.

5-3. Methods

Study sites

The field observations and experiments were conducted in 11 study rivers from 24 rivers targeted in Chapter 4 (Fig. 5-2), basing on the following three reasons: 1) the proportion of ≤ 20-years-old juveniles in population varies (0.00–0.54) and 2) the adult mussel density (1.85–40.00 individuals/m^2) has variation among the rivers, and 3) the study rivers are easily accessible. The detailed information on the conditions of study rivers is shown in Table 5-1. The name of the river and watersheds coincide with Chapter 4 (Table 4-1). Two study reaches were established in each study river in Chapter 4. In this chapter, one study reach was chosen from the two study reaches based on the accessibility to conduct the intensive observations and experiments [20.0–34.2 (mean 23.6 ± 1.40 SD) m length].

Index of adult stage: gravid density

The field observations were carried out in the study rivers two times 3–6 and 9–11 May 2018 to determine the gravidity rate of *M. togakushiensis*. These study periods are around the peak of the glochidia release of the target species (Kobayashi and Kondo 2009, K. Miura personal observation). In addition to the above two periods, the additional observation was carried out from 17 to 18 May 2018 to check whether gravid individuals existed in the study rivers where gravidity rate was not zero in the second observation occasion. This additional observation was for deciding the sampling timing of host fish to use as indicators of the parasitic stage (see next section). One investigator randomly collected visible mussels using a glass-bottom viewing bucket for 15–30 min in each study reach. Shell length of 40 mm was considered as threshold size above while individuals of target species were sexually mature in this region (K. Miura unpublished data). Thus, at least 50 individuals of *M. togakushiensis* with a more than 40-mm shell length were collected from each river. Each individual was checked for the presence of matured glochidia in the gills by slightly opening the shell valves (< 5 mm) with a shell opener. The mussels with swollen and cream-colored outer demibranchs were considered to be gravid individuals (Hastie and Young 2003,

Kobayashi and Kondo 2009). Each mussel was photographed in the same manner as methods in Chapter 4 in relation to the gravidity condition (presence/absence of matured glochidia). Each collected mussel was marked with a small sign on the shell surface using white paint or a hand drill (Negishi and Kayaba 2009, Miura et al. 2018a), and each was immediately released to the original study reach after marking. If the marked mussels were captured on the occasion of later observation, those were excluded from targets of observation to avoid handling effects. Later, shell length and height of each individual was measured using the Image J software from the photographed images as the same protocols performed in Chapter 4. Species identification of each mussel individual was performed by using simple non-lethal identification criteria developed in Chapter 3 (detailed methods are shown in Figure 3-7). Gravidity rate (the number of gravid individuals/ the total number of checked individuals) of each study river was calculated using data of individuals identified as *M. togakushiensis*. Adult density of each river was calculated using quadrat-based data obtained from field observation in Chapter 4. Gravid density (individuals/m^2) of each river was finally calculated by multiplying gravidity rate with adult density.

Indices of the parasitic stage: glochidia density

Electrofishing was performed using a backpack electrofisher (Model 12B, Smith-Root Inc., Vancouver, WA, USA) between 6–11 June 2018 to examine glochidia density. This period is a parasitic period of the target species in the study region (Kurihara and Goto 2011). Attached glochidia are perished within up to two weeks if they cannot tolerate the immunity of host fish (Ziuganov et al. 1994). Thus, the potential number of glochidia succeeded in infection can be determined by observing the host fish after two weeks since the end of the glochidia releases. The study period was at least two weeks later since the period in which the reproductive period of each river was mostly ended (< 2.5% of gravidity rate on May 17–18, 2019). One study reach having the lengths of 10 times of mean wetted-width of the study reach was established in each study river. The host fish density was estimated by two pass electrofishing method (quantitative electrofishing). The fork length (FL, mm) of each captured fish was measured after anesthetizing (FA100, DS Pharma Animal Health Co., Osaka, Japan). The number of infected larvae and infection success of margaritiferids can be affected by the differences in age of host fish [young-of-the-year (YOY) vs.

≥ 1^+ age host] (Österling 2015). YOY host fish is generally important for the recruitment of margaritiferids because the immune acquisition is not made (Österling et al. 2008, Österling and Hogberg 2014). However, encystment by host tissues and growth of glochidia on gills of ≥ 1^+ host fish were preliminarily observed in this region. Therefore, both YOY and ≥ 1^+ host individuals were collected and distinguished by the differences in fork length (Fig. 5-3, FL of YOY individuals: 30.00–60.00 mm). When the number of captured individuals of each age class in each study reach didn't reach 10 individuals at quantitative electrofishing, additional electrofishing was performed 1–2 h to qualitatively collect host fish individuals within the study reach and 50-m upstream and downstream of the study reach. Ten to 20 individuals of each age class were brought back to the laboratory and were frozen after anesthetizing. Remaining individuals were immediately released after recovering the anesthesia. Five transects were laid at the fish collected study reach and wetted-width was measured on each transect using a 50-m tape. The host density (individuals/m^2) of the YOY, and ≥ 1^+ individuals were calculated respectively using the data of quantitative electrofishing.

More than ten host fish individuals of each age class collected from each study river were dissected to count the number of infected glochidium larvae on each individual, but only two rivers had more than ten samples of YOY individuals [YOY: 1–20 (mean: 8) individuals/river in six rivers, ≥ 1^+: 2–17 (mean: 13) individuals/river]. Then, all gills (a total of eight-gill arches/fish) were removed from each individual, and the total number of infected larvae of each fish was counted under a stereomicroscope in the laboratory. Glochidia density of each study river was obtained as follows. First, the mean glochidia load (the numbers of infected individuals /fish) was separately calculated between two age classes (YOY and ≥ 1^+) because the body size of host fish was largely different between two size classes [FL: YOY, 29.00–49.00 (mean 37.94 ± 4.31 SD) mm, ≥ 1^+,69.00–330.00 (mean 125.91 ± 38.67 SD) mm, Fig. 5-3]. Second, host densities of each size class were multiplied by mean glochidia load of each age class. Third, the number of glochidia per unit area, which is defined as "glochidia density", was calculated with a direct summation of the products of host density and mean glochidia load in each of two age classes. When only one individual of YOY was captured, the glochidia load of YOY individuals was estimated from only one individual.

Index of the post-parasitic juvenile stage: juvenile survival rate

Juvenile survival rate was measured using a Buddensiek mesh cage method in the study rivers (Buddensiek 1995). This method has been used for determining the habitat preferences of juveniles and can measure juvenile survival by exposing them to actual river conditions (e.g. Denic et al. 2015, Černá et al. 2018). The improved Buddensiek mesh cages were used (Figs. 5-4a–c, 130 mm long axis, 90 mm short axis, 10 mm thickness, 5 mm cell diameters). < 250 μm particles of sediment are potentially harmful to juvenile survival (Strayer and Malcom 2012). Thus, the 250 μm mesh-nets were used for constructing mesh cages so that fine sediments could pass through the mesh and harmful effects can be tested if there were any. The juveniles were collected by artificial infection using about 500 YOY white-spotted char individuals (FL: 25.00–40.00 mm). These host fish individuals were produced by artificial insemination and cultured in indoor aquariums of Tomakomai Experimental Forest of Hokkaido University (lat. 42°40′N, long. 141°35′E). The origins of YOY individuals were six gravid females [FL: 462.00–668.00 (mean 532.83 ± 90.83 SD) mm] and 14 adult males [FL: 158.00–460.00 (mean 224.43 ± 71.97 SD) mm] collected from Watersheds *C* and *H* in autumn 2017 (D. Ito unpublished data). Six gravid mussels (shell length: 50.00–68.00 mm) were collected from River *u* for the infection on 18 May 2018. The host fish was exposed to well-aerated glochidia slurry (40,000 N/L) derived from the six gravid females for 30 minutes and kept until the excystment of juveniles on 19 May 2018. The detailed infection procedures are shown in Ito (2020). The peak of juvenile excystment occurred from 23–28 July 2018 and individuals were collected gently using Pasteur pipettes under a stereomicroscope (Fig. 5-4d). Only juveniles collected during the peak period were used for the experiments to avoid bias by using incompletely developed juveniles. In 29–31 July 2018, five live juveniles (shell length: 300–400 μm) were placed in each cell, giving a total of 30 individuals for each cage after checking juvenile survival on the basis of the visible juvenile activities (valve movement and/or foot activities) under a stereomicroscope. Triplicate of mesh cages was installed on the riverbed in similar environments as much as possible in each study river (a total of 90 individuals/river) using metal bars between 30 July and 1 August 2018 and monitored for three months (Fig. 5-4c). Each mesh cage was assessed to enumerate the number of survival of juveniles on three occasions: 27 August–1 September (1st monitoring), 27–29 September (2nd monitoring), and 22–24 October (3rd monitoring). The mussel survival was judged on the basis of the visible juvenile activities. After finishing the 3rd monitoring

occasion, all mesh cage was removed and all surviving juveniles were preserved in 75% ethanol solution. The juvenile survival rate (the number of survived juveniles/the number of initially installed juveniles) of each river was calculated on each monitoring timing.

Environmental factors

Ten environmental factors were measured in each study river; mean and maximum temperatures, total suspended solids (TSS), suspended solids of organic and inorganic fractions, pH, electrical conductivity (EC), dissolved oxygen (DO), and physical environment (water depth and current velocity) potentially affecting gravidity density, glochidia density, and juvenile survival rate. Water temperatures were continuously measured at intervals of four hours using a logger (HOBO 8K Pendant, Onset Co., Bourne, MA, USA) set in each study river from 6 May 2018 to 24 October 2018. But, one logger in the River *n* recorded water temperature only from 6 May 2018 to 17 June 2018 due to malfunction. Gravid density and glochidia density can be affected by suspended solids in the water column (see "a theoretical framework"). Thus, total suspended solids (TSS hereafter) were also measured. Surface water samples (4 L) were collected from the flow of the stream in each study river on 27–29 September 2018. The sampling period was set when ordinary water discharge was observed with no strong rain within a week in the study region. Generally, fine sediment in rivers can be more abundant in the high discharge conditions (Gordon et al. 2004). However, if the study area spreads across a large area, which was the case, it is difficult to collect the sample on equal conditions because the beginning, peak, and end of rain and the peak discharge can vary spatially. Thus, the study chose the ordinary water discharge occasion to represent the amount of fine sediment of each study river on equal conditions. Collected water samples were taken back to the laboratory for subsequent treatments. Water samples were filtered using pre-combusted and -weighed glass-microfiber filters (0.7 μm pore size). Filters were dried for at least 24 h at 60 degrees Celsius and reweighed to obtain TSS (mg/L). The effects of organic particles and inorganic particles in the river on the survival of juveniles can differ (Österling et al. 2010, Strayer and Malcom 2012). Thus, the suspended solids were separated into organic and inorganic fractions (called as fine organic and inorganic sediment, respectively hereafter) as follows. After TSS measurements, the filters were subsequently ashed for 3 h at 450 degrees Celsius and

weighed again to obtain fine inorganic sediment (mg/L); the fine organic sediment was also calculated from the differences between TSS and fine inorganic sediment.

On the monitoring period of the gravidity of mussels, fish sampling occasion, and each monitoring occasion of the field experiment, electrical conductivity (EC, mS/m) was measured (WM-32EP, TOA DKK Co., Japan) at the uppermost of each study reach. EC strongly had a positive correlation with total nitrogen (TN) and total phosphorus (TP) of river surface water (r = 0.88 and 0.45 with $p < 0.05$) in the study region (Fig. 5-5, K. Inoue and K. Miura unpublished data). Thus, EC can be used as an indicator of the nutrient concentration of river water. Nutrient concentrations can also fluctuate under different discharge conditions (Jordan et al. 1997). Thus, EC was also measured on ordinary water discharge conditions (i.e. each monitoring occasion) because of the same reason for the sampling of TSS. On each monitoring occasion of the field experiment, pH was also measured at the uppermost of each study reach by using a hand-held water quality meter (WM-32EP, TOA DKK Co., Japan). In addition, dissolved oxygen (DO, mg/L) was measured at the points where mesh cages were installed (HQ40d, Hack Co., USA).

Physical variables (i.e. current velocity and water depth) were measured immediately before checking the survival of juveniles in each river on each monitoring occasion. Velocity (cm/s), averaged over 6 s at 40 % depth from the streambed, and water depth (cm), were measured at the central point of the mesh cages using propeller-based flow meter (model CR-11; Cosmo-riken Inc., Tokyo, Japan) and a meter stick.

Statistical analyses

If any life-history stage indices obtained from field observations and experiments (i.e. gravid density, glochidia density, and juvenile survival rate) regulate the recruitment of *M. togakushiensis*, it was predicted that the indicator and the recruitment (i.e. the proportion of juveniles within the population) would have a positive correlation (e.g. Stoeckl et al. 2015, see also "A theoretical framework" in this chapter). I constructed generalized linear models (GLMs) or generalized linear mixed models (GLMMs) to test the correlation between the proportion of juveniles and each life-history stage indicator. The proportion of juveniles was used as the response variable with each life-history stage indicator as an explanatory variable. The gravidity rate of *M.*

togakushiensis at the first monitoring [0.00–0.51 (mean 0.31 ± 0.17 SD)] was higher than the second [0.00–0.44 (mean 0.14 ± 0.15 SD)] (GLM with log-likelihood ratio tests, $p < 0.01$, Fig. 5-6). The lower gravidity rate on 2nd monitoring means that the glochidia release had already occurred and thus measured values did not necessarily represent true gravidity rate. Therefore, the values of the gravidity rate of the first monitoring were used for the calculation of gravid density. For models with the juvenile survival rate as an explanatory variable, the timing of monitoring (1st, 2nd, and 3rd) was used as a random factor. Then, the values of juvenile survival rate of 2nd and 3rd monitoring in the River *i* was excluded from model construction because all experimental cage was buried in the streambed at the depth of more than 30 cm that was too deep for the habitat of juveniles between the end of 1st monitoring and the 2nd monitoring.

When two or more indicators of life-history stages had positive relationships with the proportion of the juveniles, I constructed GLM with the proportion of the juveniles as response variables. The proportion of the juveniles had a positive relationship with glochidia density and juvenile survival rate (GLM with log-likelihood ratio tests, $p < 0.01$). Thus, glochidia density, the mean juvenile survival rate of three monitoring timing, and their interaction (see "Results") were used for explanatory variables. Then, the juvenile survival rate of the River *i* was represented by the value of 1st monitoring. The explanatory variables were standardized and the significance of each explanatory variable was tested using the Wald Z statistics determining the relative relationship between two key life-history stages.

To find the limiting factors of glochidia density, the relationship between glochidia density, gravid density, EC, TSS, and mean temperature during the parasitic periods of *M. togakushiensis* (6 May to 17 June 2018), I constructed GLMs with glochidia density as the response variable. Before the construction of the models, the pairwise correlations among four factors were conducted using Pearson's correlation tests to check the presence of multicollinearity ($r > 0.60$ with $p < 0.05$). There was no correlation among four explanatory variables ($r < 0.60$ with $p > 0.05$). Thus, I used four factors as explanatory variables. I developed all possible models by removing each of the explanatory variables progressively, and multiple models were compared based on Akaike's Information Criterion (AIC, Akaike 1974). I considered models with $\Delta AIC < 2$ to be meaningful representations of the relationship between explanatory variables and response variables

(Burnham and Anderson 2002). Model averaging was performed for models with $\Delta AIC < 2$. In each averaged model, explanatory parameters for which the 95% confidence intervals (CI) did not include zero were considered influential parameters.

I constructed GLMMs with juvenile survival rate as response variables to determine the limiting factors of juvenile survival rate. Before the construction of the models, I examined pairwise correlations between response variable (i.e. juvenile survival rate) and nine potential environmental factors (mean and maximum temperatures during the experiments, fine organic and inorganic sediments of, pH, EC, and mean dissolved oxygen, and mean water depth and velocity at the three points of experimental cages) by using Pearson's correlation tests. EC and fine sediment (both organic and inorganic fractions) were relatively well correlated with response variable ($r > 0.50$ with $p < 0.01$). But, fine organic and inorganic sediments were strongly correlated to each other ($r = 0.91$ with $p < 0.01$). Fine inorganic sediment had a higher correlation with response variable ($r = -0.63$ with $p < 0.01$) than that of organic fractions ($r = -0.57$ with $p < 0.01$) and there was no multicollinearity between fine inorganic sediment and EC ($r = 0.08$ with $p = 0.66$). Thus, fine inorganic sediment, EC, and interaction of the two variables were used as explanatory variables to construct models. Monitoring timing (1st, 2nd, or 3rd) was used for a random factor. Then, juvenile survival rates of the river i on 2nd and 3rd monitoring were not included in the models as with reasons the above model construction. The model selection was also conducted in the same manner as above. But, model significance was tested using log-likelihood ratio tests against null model because models with $\Delta AIC < 2$ (i.e. top model) was only one model as a result of the model selection. I reconstructed the top model after standardizing explanatory variables and the significance of each explanatory variable was tested using the Wald Z statistics.

In GLMs and GLMMs, binomial error distribution was used with a log link function for models with ratio data as a response variable. In addition, Gaussian error distribution was used for other models. For the models with Gaussian error distribution, log-transformation of the response variable was performed when needed to improve normality. All statistical analyses were conducted using R 3.0.3 (R Development Core Team 2015) with a significance level of $\alpha = 0.05$. The packages of glmmADMB (Fournier et al. 2012), lme4 (Bates et al. 2017), and MuMIn (Barton 2011) were used for fitting and comparing models.

5-4. Results

The gravidity of a total of 1260 individuals of *M. togakushiensis* was checked on the two monitoring occasions. The number of monitored individuals of each study river ranged from 31 to 81 (mean: 57) individuals/river. The gravidity rate of *M. togakushiensis* on 1st monitoring was higher than those on the 2nd monitoring occasion (GLM with log-likelihood ratio tests, $p < 0.01$, Fig. 5-6). The model that has a gravid density as an explanatory variable significantly differed from the null model, but the relationship between the proportion of juveniles and gravid density was significantly negative (GLM with log-likelihood ratio tests, $p < 0.01$, Table 5-2a).

A total of 277 host fish individuals were collected. Among the collected host fish individuals, 90 YOY [1–64 (mean 15.00 ± 24.27 SD) individuals/river] and 187 ≥ 1^+ individuals [2–27 (mean 17.00 ± 6.81 SD) individuals/river] were collected from six and all study rivers, respectively (Table 5-3). Three individuals collected from the River *a* (Watershed *A*) were *S. malma kraschenninnikovi* and the others were *S. leucomaenis leucomaenis*. All *S. malma kraschenninnikovi* individuals were infected by larvae of *M. togakushiensis* [369–615 (mean 476.3 ± 102.8 SD) individuals/fish] and glochidia were encysted. Thus, those individuals were included in the following analyses. Of the collected fish, a total of 191 individuals (48 and 143 individuals of YOY and ≥ 1^+) of host fish individuals were dissected and their gills were observed to count the number of glochidia infected (i.e. glochidia load) (Table 5-3). The infection of *M. togakushiensis* larvae was confirmed from the host samples of all study rivers. The glochidia load per fish was 0.00–253.00 individuals/fish (mean 64.26 ± 55.06 SD) of YOY and 0.00–8319.00 (mean 727.52 ± 1381.94 SD) individuals/fish of ≥ 1^+, respectively. The mean glochidia load of YOY individuals ranged between 15.00–134.00 (mean 65.98 ± 41.29 SD) among six study rivers and of ≥ 1^+ individuals ranged between 130.24– 2045.31 (mean 830.30 ± 753.88 SD) among 11 study rivers (Table 5-3). The glochidia density of each river ranged between 3.17–65.51 (mean 29.81 ± 22.83 SD) individuals/m^2 (Table 5-4). The model that glochidia density as an explanatory variable significantly differed from the null model, and glochidia density was positively correlated with the proportion of juveniles (GLM with log-likelihood ratio tests, $p < 0.01$, Table 5-2a). The top eight models include EC, gravid density, mean temperature, and TSS (Table 5-5a). The 95% confidential intervals of all factors include zero,

indicating non-meaningful (Table 5-5b).

Experimental mesh cages were not broken and dislodged for three months of the field experiments. The juvenile survival rates ranged between 0.00–0.78 (mean 0.39 ± 0.30 SD), 0.00–0.74 (mean 0.35 ± 0.27 SD), 0.00–0.64 (mean 0.28 ± 0.24 SD) on 1st, 2nd, and 3rd monitoring, respectively. The juvenile survival rate gradually decreased along with the progression of the time (Fig. 5-7). The model that juvenile survival rate as an explanatory variable significantly differed from the null model, and the juvenile survival rate was positively correlated with the proportion of juveniles (GLM with log-likelihood ratio tests, $p < 0.01$, Table 5-2a). The top model that includes fine inorganic sediment, EC, and their interaction as explanatory variables was significant (GLM with log-likelihood ratio tests, $p < 0.01$, Table 5-6a). The signs of the three explanatory variables were negative. The effects of fine inorganic sediment, EC, and their interaction on the juvenile survival rate were significant (Wald Z statistic, $p < 0.01$, Table 5-6b). The juvenile survival rate was interactively decreased by fine inorganic sediment and EC (Fig. 5-8).

The effects of mean juvenile survival rate and the interaction of mean juvenile survival rate and glochidia density on the proportion of juveniles were significant (Wald Z statistic, $p < 0.01$, Table 5-2b). The mean juvenile survival rate was smaller in rivers with lower glochidia density, and *vice versa* (Fig. 5-9).

5-5. Discussion

The results in this chapter demonstrated that habitat deterioration can cause recruitment failure of long-lived freshwater pearl mussel, *M. togakushiensis*, by reducing the survival rate of post-parasitic juveniles. It was also indicated that the post-parasitic juvenile stage is the most sensitive stage in the deteriorated habitats as suggestively pointed out in the previous studies (Bauer 1988, Österling et al. 2008, 2010, Strayer and Malcom 2012). In addition, the lack of the potential number of juvenile supplies (i.e. low glochidia density) likely causes recruitment failure interactively with the juvenile survival rate. Furthermore, the lack of the potential number of juvenile supplies can cause recruitment failure even the habitat quality is high. The gravid density as an indicator of the potential amount of larval production had a negative relationship with the

proportion of juveniles. It is unclear why the gravid density showed a negative relationship with recruitment. However, it is unlikely that lower larval production causes higher reproductive efficiency because sufficient amounts of larvae are necessary for their recruitment (McLain and Ross 2005). Therefore, recruitment of *M. togakushiensis* was mainly limited at the post-parasitic juvenile stage and parasitic stages in their life histories. Overall, recruitment of *M. togakushiensis* can be limited by processes in not only one life-history stage, but also multiple stages.

Previous studies have demonstrated that the extinction debt of organisms has been caused by habitat destruction and fragmentation, and those mechanisms have been clarified at the meta-population-level process (Hylander and Ehrlén 2013). On the other hand, the theoretical studies have pointed out that habitat deterioration can also cause the extinction debt at the individual-level process. Specifically, if one or several life-history stages of individuals are resistant to deteriorated habitat, population extinction can be delayed (Hylander and Ehrlén 2013). However, the individual-level process of extinction debt remains poorly explored so far (Figueiredo et al. 2019). The case in this chapter provided a novel and important piece that fills this knowledge gap. Population extinction of long-lived freshwater organisms can be delayed by habitat deterioration because adult individuals can persist in deteriorated habitats while early life-history stage individuals are not viable under the low habitat quality.

The juvenile survival rate had a positive relationship with recruitment. When evaluating recruitment conditions, many studies have used the proportion of juveniles within the population as an alternative indicator of juvenile survival rate (Österling et al. 2010, Strayer and Malcom 2012). However, the causal relationship between these two had not been tested. The results provided insights into the causal mechanisms that were overlooked in previous studies by the field experiments that directly exposed the young juveniles to the actual river environment. The possible reasons that the juvenile survival rate for three months can explain ≤ 20-year-juvenile abundances are as follows. The juvenile survival rate might be lowest in the earlier juvenile stage than the later of the juvenile stage, as suggestively shown in the previous studies (Buddensiek 1995). My experiment used just metamorphosed juveniles (within a week since the detachment from host fish) thereby allowing me to detect the effects of environmental conditions on the very initial stage of the life cycle of mussels in a realistic environment in the field. The results also showed that

average 40% juvenile survival rates were almost preserved until the end of the field experiment since the first monitoring (Fig. 5-7). These imply that the juvenile survival rate might become lower at an earlier stage than a later stage in their post-parasitic juvenile stage, and this stage can limit the subsequent proportion of juveniles.

The juvenile survival rate was negatively affected by EC (an alternative indicator of nutrient concentration), fine inorganic sediment, and their interactions. Nutrients (indicated by EC) generally have no strong acute toxicity but can have weak or chronic toxicity to mussel juveniles. For example, high concentrations of $N\text{-}NO_3^-$, which is a major component of nutrients in the river water and have a strong positive relationship with EC in my study region ($r = 0.86$ with $p < 0.01$, N = 24 rivers), can reduce the juvenile survival rate (Douda 2010). This direct effect of nutrients can explain the negative effects of higher EC values on the juvenile survival rate in the study rivers. Excessive fine inorganic sediment of the water column can also directly decrease the survival rate of juvenile mussels by clogging their gills (Österling et al. 2010). In addition, when relatively heavy particles of fine sediment deposit onto the riverbed can clog the interstitial spaces of the streambed (Fung and Ackerman 2019), which potentially disrupts the water exchange of surface—hyporheic interfaces. This disruption of the water exchange can cause hypoxic conditions and higher concentrations of un-ionized ammonia that has strong toxicity to juvenile mussels, in the riverbed sediment, thereby indirectly decreasing the juvenile survival rate (Wood and Armitage 1997, Augspurger et al. 2003, Strayer and Malcom 2012). Furthermore, the deposit of fine sediment and increases in nutrient concentration can synergistically increase the negative influences on juvenile survival. Increasing nutrient concentration can increase the interstitial ammonia concentration because the loading of reactive nitrogen as the source of ammonia becomes more abundant (Strayer and Malcom 2012). The negative effects of higher nutrient concentration, fine inorganic sediment, and their interaction on juvenile mussel survival can be caused by those processes.

Glochidia density was found to be important for recruitment of *M. togakushiensis* together with the juvenile survival rate. The lack of the number of juvenile supplies to the population may eventually lead to decreasing the number of surviving juveniles. On the other hand, glochidia density didn't have clear relationships with abiotic and biotic factors, suggesting that glochidia

density is not strongly limited by those factors. The glochidia density is a function of host density and glochidia load per fish. Low habitat quality can reduce host fish availability and infection success of glochidia larvae (Beussink 2007, Österling 2019). However, there were no obvious relationships between host density, TSS, and nutrient concentrations in the study region (GLM with log-likelihood ratio tests, $p = 0.24$ and $p = 0.18$, N. Ishiyama and K. Miura unpublished data). In addition, the relationship between glochidia load of both age classes (YOY and $\geq 1^{+}$) and, TSS and nutrient concentrations were also unclear (GLM with log-likelihood ratio tests, YOY: $p = 0.92$ and $p = 0.38$, $\geq 1^{+}$: $p = 0.24$ and $p = 0.62$). Therefore, the effects of habitat deterioration on mussel recruitment of *M. togakushiensis* indirectly through the effects on host population size are small in the study region.

The study in this chapter empirically demonstrated that extinction debt (i.e. recruitment failure) can be caused by habitat deterioration at the individual-level process. These results suggested that if habitat quality is low for surviving early life-history stage individuals but adult individuals can persist, extinction debt can be caused even though population size is large in unfragmented and large habitat. Therefore, when there are some possibilities of habitat deterioration, managers need to pay attention to the sensitivity of the early life-history stage individuals. As far as possible, it is effective to test the survival rate of early life-history stage individuals (e.g. seedling emergence of tree species).

Fine inorganic sediment and nutrients are generally related to the land-use in the catchments (Wood and Armitage 1997, Gordon et al. 2008, Howarth et al. 2012). The land-use in the catchment of the 24 study rivers targeted in Chapter 4 (including the 11 study rivers of Chapter 5) was additionally calculated by using the methods of "GIS analysis" in Table 5-1. In the study region, EC had strong positive relationships with agricultural land and urban covers in the catchments (Fig. 5-10, N = 24 rivers, $r = 0.73$ and 0.86 with $p < 0.01$), which means that the nutrients were derived from mainly agricultural land and urban area. On the other hand, fine inorganic sediment had no clear relationship with land-use in the catchment. Fine inorganic sediment is also likely related to the agricultural land cover (Wood and Armitage 1997). The most suspicious source of fine inorganic sediment in the study region is agricultural land because of the high covering area ratio in the catchments. However, there was no apparent relationship between fine inorganic

sediment and agricultural land cover in the catchments (N = 24 rivers, r = 0.02 with p = 0.92). The study has not yet determined the reasons for the variation in the amount of fine inorganic sediment.

The overall results of the processes of recruitment failure of *M. togakushiensis* in the study region are shown in Figure 5-11. In summary, the proportion of juveniles can be limited by the interaction between glochidia density and juvenile survival rate. Glochidia density is not limited by environmental factors. Juvenile survival rates can be interactively reduced by increased nutrient concentration and fine inorganic sediment. Nutrients were derived from agricultural land and urban area in the catchments. The relationship between the amount of fine inorganic sediment and land-use in the catchment was unclear. In the next chapter, I summarized the results of this study and discussed what ecosystem management and conservation measures are important for recruitment of freshwater mussel species including *M. togakushiensis*.

Table 5-1 The summary of basic information on conditions of the 11 intensive study rivers

Watershed area	River	Adult mussel density (N/m²)	Prop. of juv. inds.	Max. age (yr)	Min. age (yr)	Depth (cm)	Velocity (cm/s)	Width (m)	Prop. of agricultural land**	Prop. of wetland**	Prop. of urban area**
A	*c*	6.67	0.26	>100	13.14	24.78	19.8	5.22	54.27	3.95	1.89
B	*d*	23.33	0.16	68.53	0.12	36.10	25.9	5.82	69.46	6.99	1.08
C	*e*	37.03	0.03	63.22	11.15	30.47	31.6	6.81	70.07	4.45	0.72
D	*f*	24.44	0.03	>100	10.76	14.50	19.1	2.81	68.78	6.88	0.52
E	*i*	11.11	0.20	96.17	7.34	22.84	27.6	5.28	68.29	5.18	1.02
G	*n*	1.85	0.22	>100	7.84	47.44	20.7	7.67	67.32	12.01	0.59
	p	5.18	0.00	>100	25.98	38.03	26.0	3.87	37.30	8.93	0.05
	q	17.04	0.00	72.46	24.32	28.80	36.6	3.84	27.51	28.56	0.11
H	*u*	40.00	0.10	79.03	3.42	19.10	20.8	3.00	15.26	0.57	0.00
	w	11.85	0.00	>100	28.35	42.87	30.4	5.01	56.58	4.35	0.80
	x	14.07	0.54	>100	3.99	38.77	21.2	6.62	52.52	20.21	0.28

*Proportion of juvenile individuals: the proportion of the ≤ 20-years-old juveniles in the population

** The proportion of land-use types in the catchment calculated by the "GIS analysis" below

GIS analysis.—The study derived watershed areas for each sampling point (i.e. study reach) using the Spatial Analyst Toolkit in ArcMap 10.6 (ESRI inc., Redland. CA, USA) with a 50-m digital elevation model (DEM) from the Geospatial Information Authority of Japan. I obtained land cover (100-m resolution) for the study region from the nation-wide land use census data (land use database in 1987, Ministry of Land, Infrastructure, Transport and Tourism). The present land cover in the study region was developed by the late 1980s (Nagasaka 2017). The land use database in 1987 has mostly no change in the current land cover

Table 5-2 (a) Summary of the results of generalized linear models (GLMs) or generalized linear mixed models (GLMMs) using the proportion of juveniles of *M. togakushiensis* as response variables and each indicator of life-history stage as explanatory variable. The model significance of each model was tested using the log-likelihood ratio test. (b) Summary of the results of GLM using the proportion of juveniles of *M. togakushiensis* as response variables and glochidia density, mean juvenile survival rate, and their interaction as explanatory variables. The significance of each explanatory variable was tested using the Wald Z statistic

a) Life-history stage/explanatory variable	Sign of coefficient	*p*
Adult stage		
Gravid density	-	< 0.01
Parasitic stage		
Glochidia density	**+**	**< 0.01**
Post-parasitic juvenile stage		
Juvenile survival rate	**+**	**< 0.01**

b) Explanatory variable	Sign of coefficient	*p*
Glochidia density	+	
Mean juvenile survival rate	**+**	**< 0.01**
Glochidia density × Mean juvenile survival rate	**+**	**< 0.01**

Bold type indicates statistical significance at $\alpha = 0.05$ with a positive relationship with response variable (the proportion of the juveniles)

Table 5-3 The summary of the information on indicators relating to the parasitic stage of *M. togakushiensis*

River	The number of collected host fish (≥ 1⁺ (YOY))	The number of dissected host fish (≥ 1⁺(YOY))	Mean glochidia load of YOY host fish	Mean glochidia load of ≥ 1⁺ host fish
c	11 (0)	11 (0)	-	384.00
d	2 (8)	2 (8)	80.88	1689.50
e	24 (0)	14 (0)	-	1282.79
f	18 (17)	15 (17)	40.88	131.47
i	17 (0)	13 (0)	-	2045.31
n	17 (0)	17 (0)	-	130.24
p	14 (1)	9 (1)	15.00	534.22
q	23 (1)	17 (1)	48.00	302.94
u	18 (64)	16 (20)	77.15	173.13
w	15 (0)	15 (0)	-	533.60
x	27 (1)	14 (1)	134.00	1926.14

Table 5-4 The results of major indicators of life-history stages

River	Prop. of juveniles*	Gravid density (N/m^2)	Glochidia density (N/m^2)	Mean survival rate of juveniles
c	0.26	0.65	10.59	0.07
d	0.16	8.75	14.59	0.10
e	0.03	16.79	33.47	0.47
f	0.03	12.40	31.94	0.60
i	0.20	4.00	62.11	0.30**
n	0.22	0.00	3.17	0.53
p	0.00	2.28	28.81	0.00
q	0.00	5.32	17.09	0.43
u	0.10	18.51	65.51	0.53
w	0.00	1.48	3.84	0.00
x	0.54	4.34	56.76	0.70

*Proportion of juvenile individuals: the proportion of the ≤ 20-years-old juveniles in the population

** The mean juvenile survival rate of the river is represented by using the value of 1st monitoring because the cages were buried in the sediment at the deep depth

Table 5-5 (a) Summary of model selection related gravid density, EC, mean temperature, and TSS to glochidia density of *M. togakushiensis* using generalized linear models (GLMs). (b) Model-averaged parameters and associated standard error (SE) for explanatory variables used in (a)

(a) Model	*K*	*AIC*	*ΔAIC*	*w*
gravid density, mean temperature, TSS	5	101.20	0.00	0.16
gravid density, TSS	4	101.83	0.63	0.11
gravid density	3	101.84	0.64	0.11
gravid density, mean temperature	4	102.29	1.09	0.09
null	2	102.98	1.78	0.06
EC, gravid density, TSS	5	103.12	1.92	0.06
EC, gravid density, mean temperature, TSS	6	103.13	1.93	0.06
TSS	3	103.16	1.96	0.06
EC, TSS	4	103.20	2.00	0.06
EC, gravid density	4	103.28	2.08	0.05
EC	3	103.28	2.08	0.05
EC, gravid density, mean temperature	5	104.20	3.00	0.03
mean temperature	3	104.98	3.78	0.02
mean temperature, TSS	4	105.14	3.94	0.02
EC, mean temperature, TSS	5	105.20	4.00	0.02
EC, mean temperature	4	105.24	4.05	0.02

(b) Variable	Model averaging results			
	β	SE	Lower CI	Upper CI
EC	-0.40	0.73	-2.20	1.41
gravid density	2.09	1.04	-0.34	4.53
mean temperature	16.97	11.12	-10.16	44.10
TSS	-0.67	0.41	-1.64	0.31

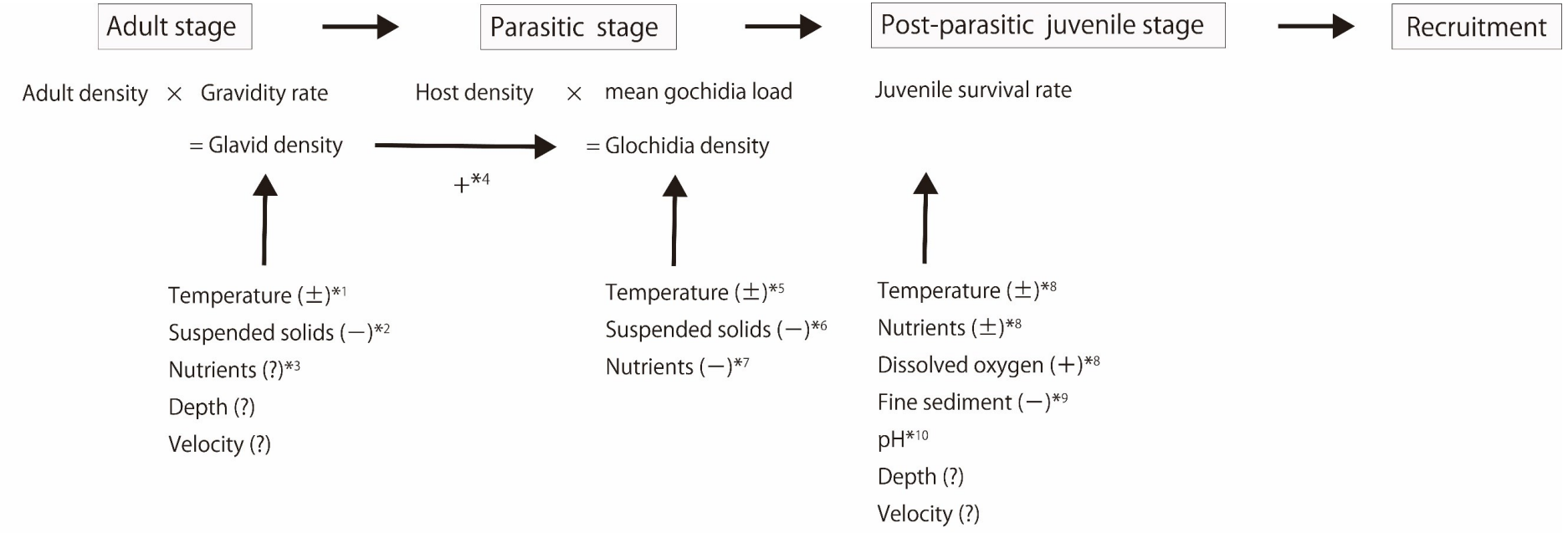
K number of parameters in the model, *AIC* Akaike's information criterion, *ΔAIC* difference in AIC value for a particular model when compared with the top-ranked model, *w* AIC model weight

Table 5-6 (a) Summary of model selection to relate environmental factors (EC and fine inorganic sediment) to juvenile survival of *M. togakushiensis* using generalized linear mixed models (GLMMs). (b) Estimated coefficients and standard errors (SE) for standardized explanatory variables used in (a). Model significance (p) was tested with the Wald Z statistics

(a) Model	K	AIC	ΔAIC	w	p
EC, fine inorganic sediment, EC × fine inorganic sediment	4	516.65	0.00	1.00	< 0.01
EC, fine inorganic sediment	3	556.97	40.31	0.00	
fine inorganic sediment	2	769.74	253.09	0.00	
EC	2	922.81	406.16	0.00	
Null	1	1168.87	652.22	0.00	

(b) Variable	Coefficient	SE	p
EC	-0.82	0.06	< 0.01
fine inorganic sediment	-1.16	0.08	< 0.01
EC × fine inorganic sediment	-0.56	0.09	< 0.01

K number of parameters in the model, AIC Akaike's information criterion, ΔAIC difference in AIC value for a particular model when compared with the top-ranked model, w AIC model weight



*1 Hastie and Young (2003), *2 Gascho Landis et al. (2013), Gascho Landis and Stoeckel (2016),*3 Bauer (1988), *4 inferred from Osterling et al. (2010),
*5 Akiyama and Iwakuma (2007), Taeubert et al. (2014) *6 Beussink (2007), *7 Moore and Bringolf (2018), *8 Buddensiek (1995), *9 Osterling et al. (2008), (2010), *10 Wang et al. (2008)

Fig. 5-1 A theoretical diagram showing the key life-history stages and indices relating freshwater mussel recruitment, and environmental factors that potentially affect each indicator. Each asterisk with a number indicates cited articles

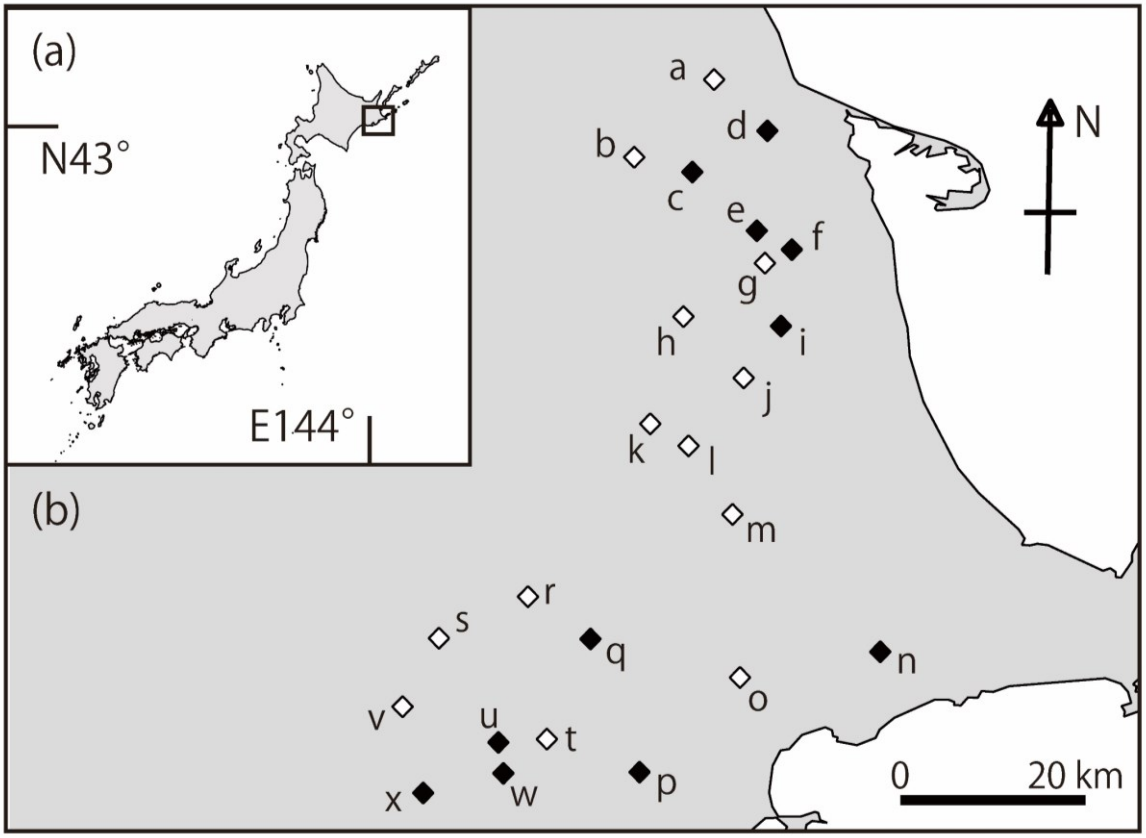


Fig. 5-2 The geographical location of the 24 study rivers including 11 intensive study rivers. White and black diamonds denote non-intensive and intensive rivers, respectively

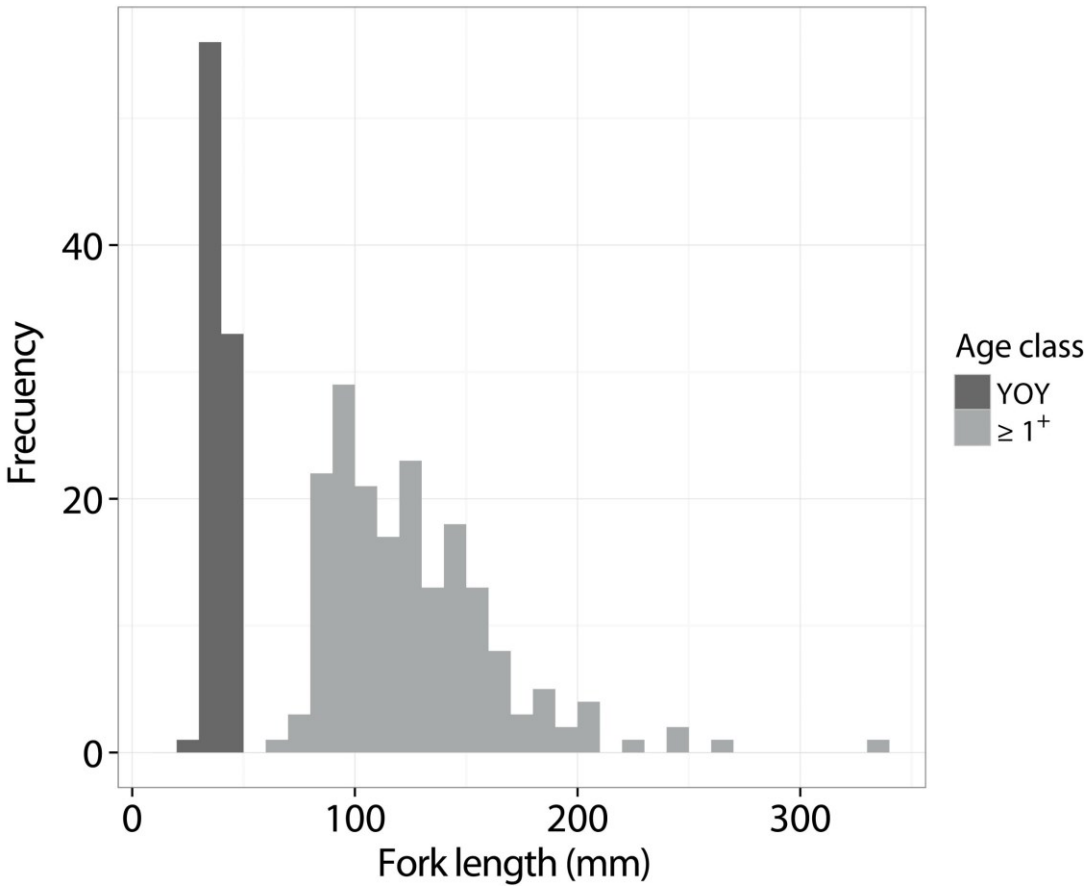


Fig. 5-3 The histogram of fork length (mm) of host fish (*Salvelinus leucomaenis leucomaenis*, three ≥ 1^+ individuals of *S. malma krascheninnikovi* are contained). Dark gray and gray indicate YOY (young-of-the-year) and ≥ 1^+ individuals, respectively

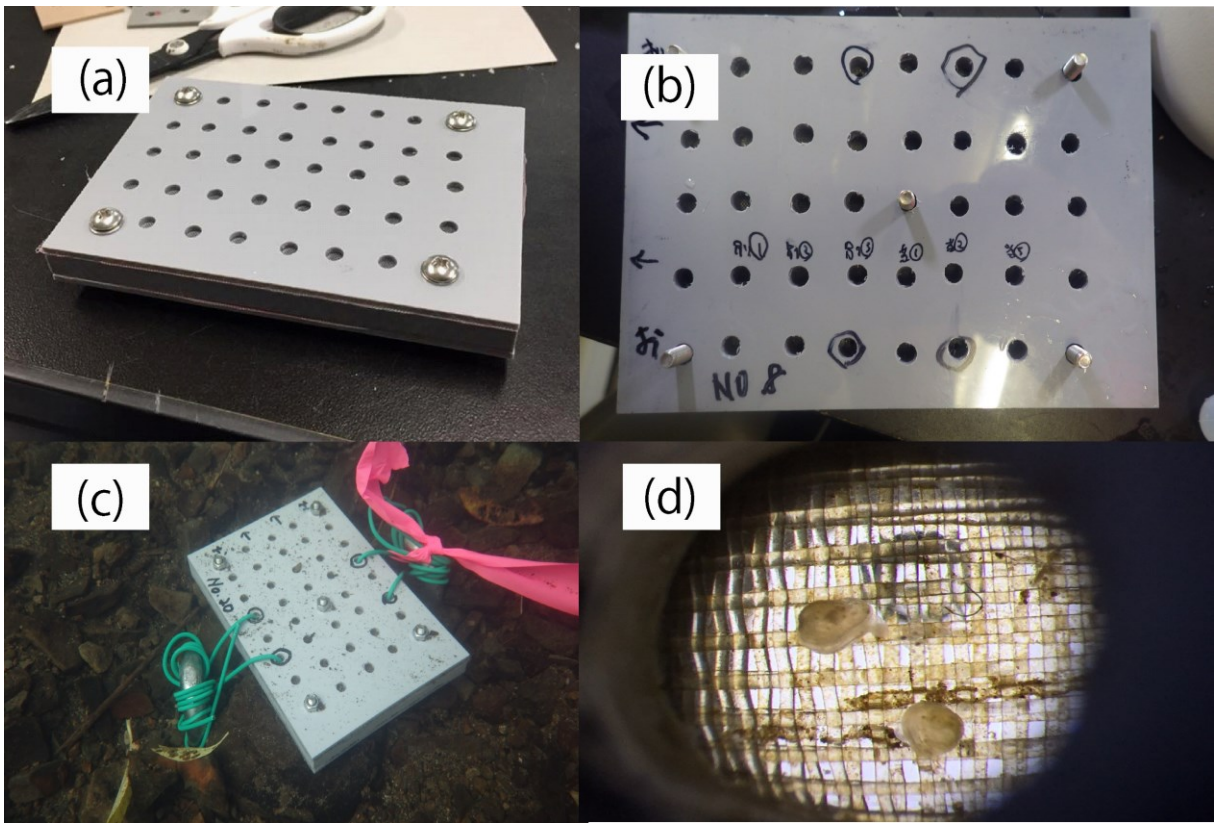


Fig. 5-4 Images of experimental mesh-cage (a), (b), and (c). An example of *M. togakushiensis* juveniles in a cell of experimental cage observed using a stereomicroscope (d)

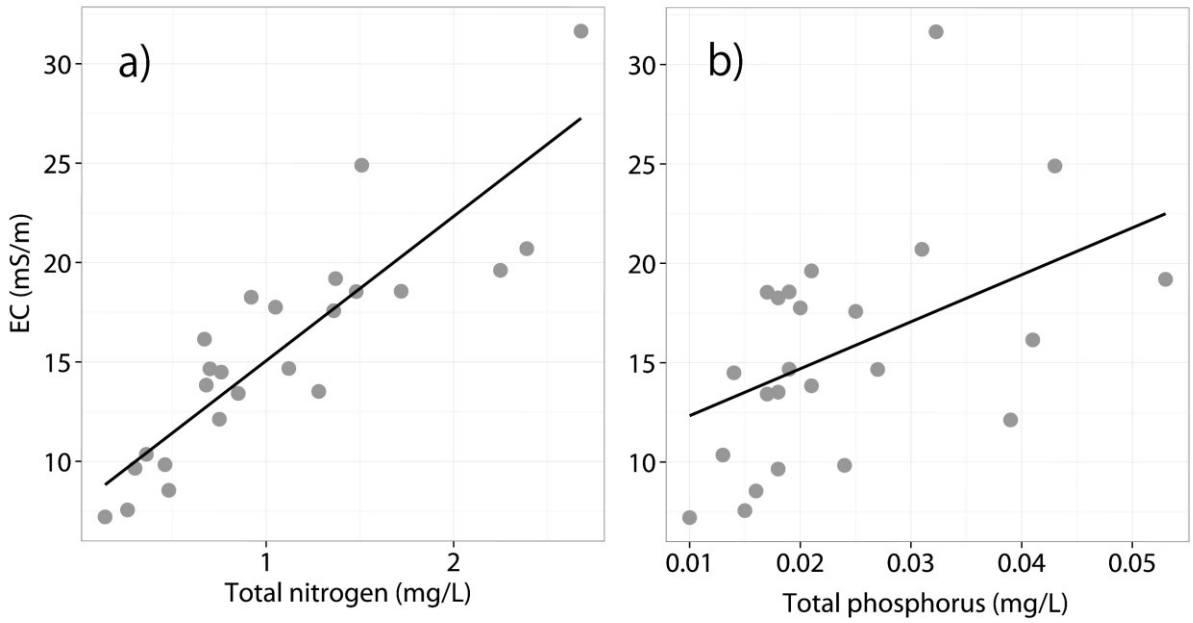


Fig. 5-5 Relationships between electrical conductivity (EC) and total nitrogen (a) and total phosphorus (b) concentration. The black lines were drawn using the regression in the generalized linear models (GLM) with a Gaussian error distribution

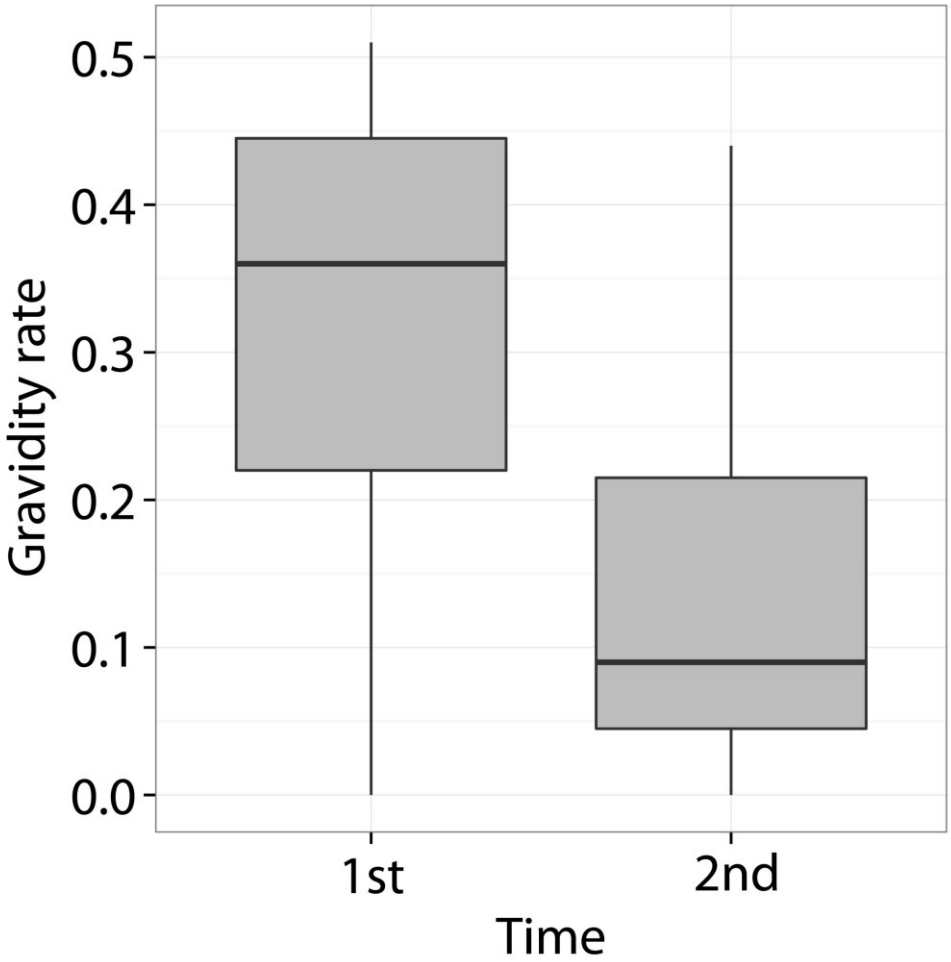


Fig. 5-6 Boxplots showing gravidity rate of *M. togakushiensis* on 1st and 2nd sampling occasions

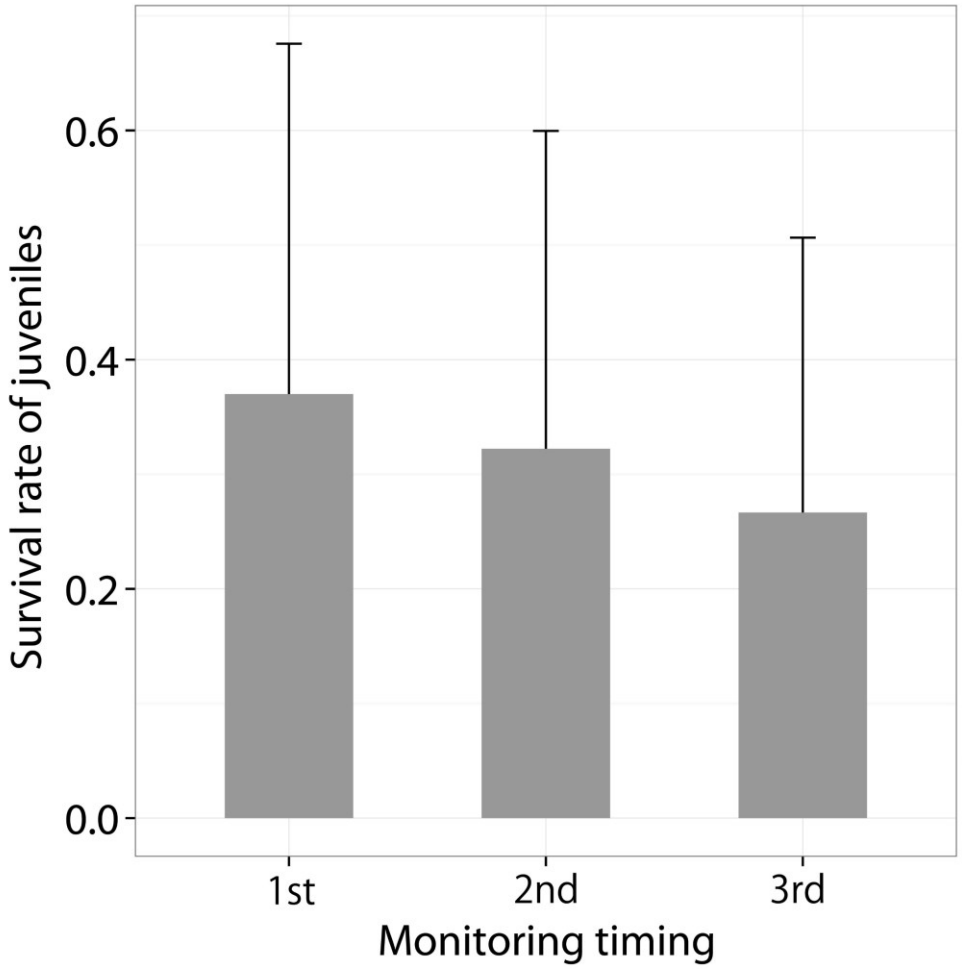


Fig. 5-7 The bar plots of time changes in the juvenile survival rate. Error bars show standard deviations

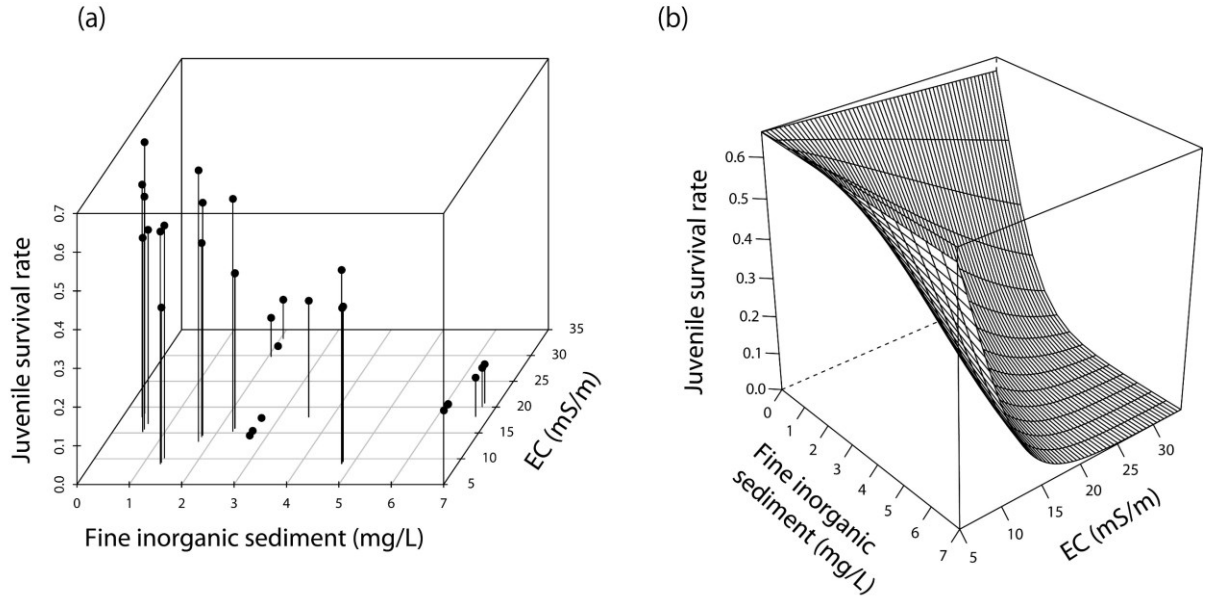


Fig. 5-8 Interactive effects of the juvenile survival rate of *M. togakushiensis*, Electrical conductivity (EC, ms/m), and fine inorganic sediment (mg/L). Actual measurements of the variables (a). Estimates of the juvenile survival rate using the generalized linear mixed model (GLMM) with a binomial error distribution (b)

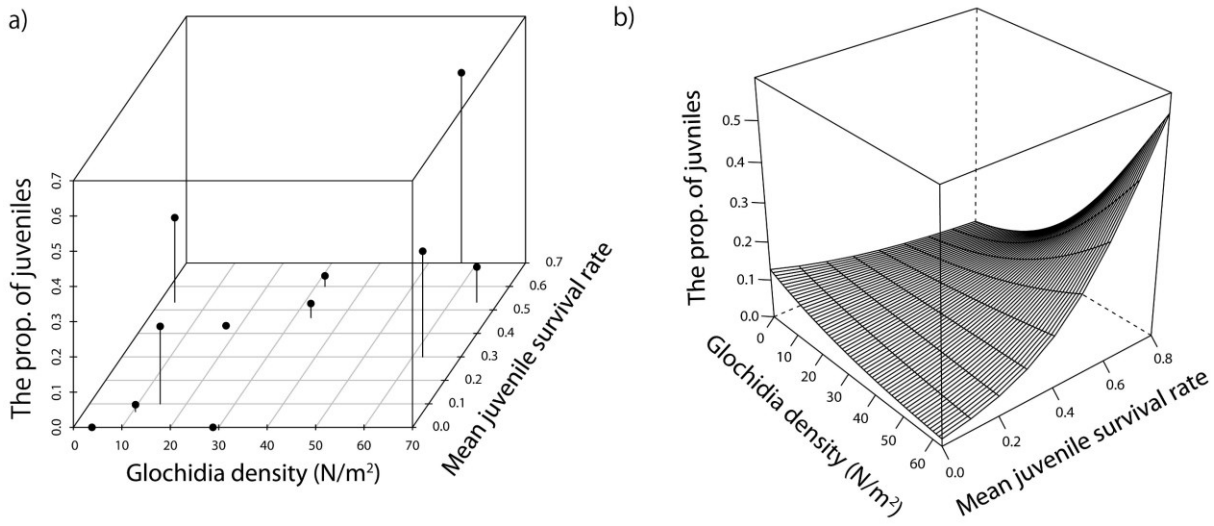


Fig. 5-9 Interactive effects of the proportion of the juveniles of *M. togakushiensis*, mean juvenile survival rate, and glochidia density (N/m²). Actual measurements of the variables (a). Estimates of the proportion of the juveniles using the generalized linear model (GLM) with a binomial error distribution (b)

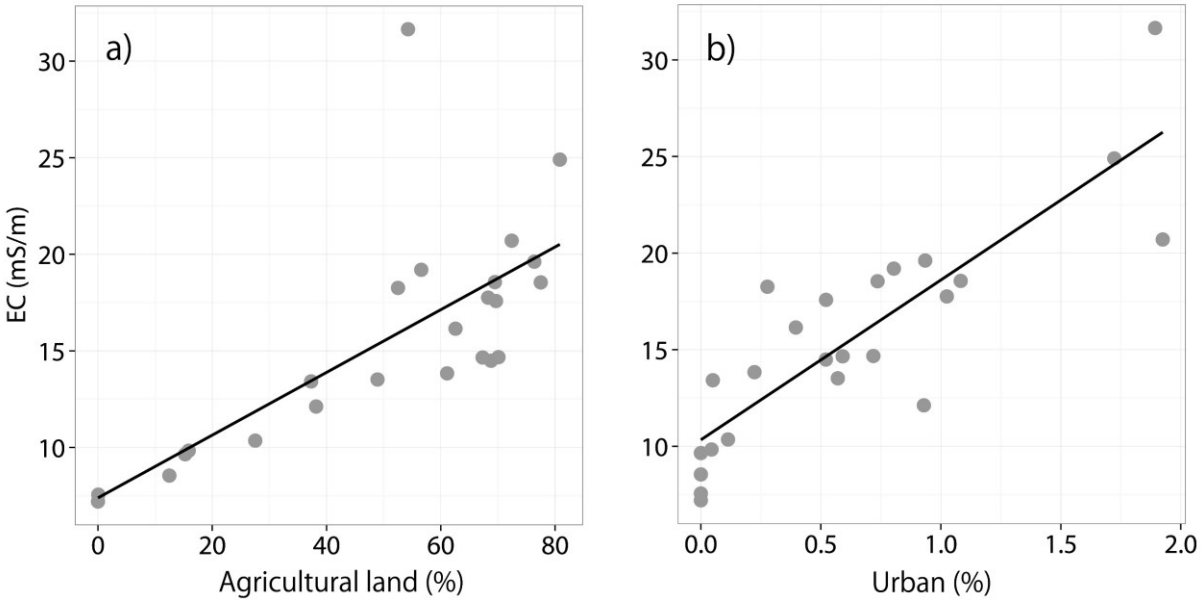


Fig. 5-10 Relationships between electrical conductivity (EC, mS/m), and agricultural land (a) and urban (b) covers (%). The black lines were drawn using the regression in the generalized linear models (GLM) with a Gaussian error distribution

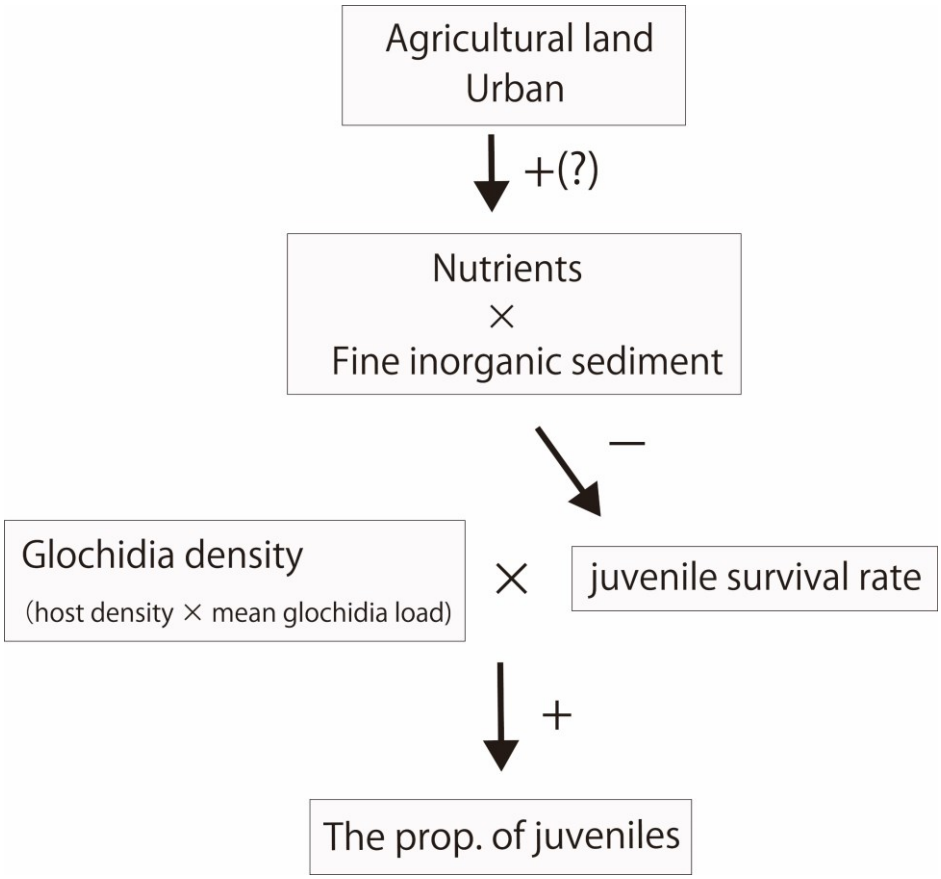


Fig. 5-11 Graphical results of the process of the recruitment failure of *M. togakushiensis* in the study region. The arrows indicate the direction of the major processes involved

6. General discussion

In this chapter, I comprehensively discussed the importance of the results of the thesis for the future conservation of *M. togakushiensis* in the study region and other long-lived freshwater mussels in the world. In addition, I discussed types of possible conservation measures including catchment-scale management and captive propagation that are considered to be effective to prevent the extinction of *M. togakushiensis* populations in eastern Hokkaido.

The major results of this thesis can be summarized as follows. First, *M. togakushiensis* was likely to co-occur with *M. laevis* at river reach scale (within 50–100 m) in Hokkaido. In addition, co-occurrence distributions of two pearl mussel species and populations of *M. togakushiensis* were relatively well confirmed in eastern Hokkaido (Chapter 2). In response to the high possibilities of co-occurrence distributions of two pearl mussel species, I developed simple non-lethal identification criteria of two pearl mussel species (Chapter 3). These criteria used outer shell morphologies (i.e. length and height) and identified nonlethally a number of collected pearl mussel individuals with high accuracies in eastern Hokkaido (86–96% with 92% mean). Using the identification criteria developed in Chapter 3, I investigated the status of 24 populations of *M. togakushiensis* in eastern Hokkaido (Chapter 4). A total of seven rivers were considered as "functional" with more than 20% of individuals being ≤ 20-year-old (i.e. juveniles). I also showed the recruitment failure of *M. togakushiensis* in four rivers where populations completely lacked juveniles. Maximum estimates of time remained until the extinction of those populations, which was based on age distributions, were approximately 70 years. Finally, I demonstrated the processes of recruitment failure (i.e. extinction debt) of *M. togakushiensis* populations (Chapter 5). Recruitment failure of *M. togakushiensis* could be caused by excessive fine sediment load and nutrient inputs. The survival rate of the post-parasitic juvenile stage could be impaired by habitat deterioration. In addition, sufficient potential number of juvenile supplies at the parasitic stage is necessary for the recruitment even when the juvenile survival rate is high. These results mean that the recruitment of *M. togakushiensis* can be limited by the multiple reproductive life-history stages.

The most important finding of this thesis was that the processes of recruitment failure of long-lived freshwater mussel species were elucidated in relation to major multiple life-history stages. The previous studies have targeted on a part of life histories of freshwater mussel species

when determining the cause of recruitment failure (Österling et al. 2008, 2010, Strayer and Malcom 2012, Österling and Hogberg 2014, Haag et al. 2019). However, it was difficult to recover the natural recruitment of freshwater mussel species when targeting only a part of life histories under such situations (Galbraith et al. 2018). My results empirically supported the hypothesis that the post-parasitic juvenile stage is the most sensitive to habitat deterioration among major reproductive life-history stages of freshwater mussel species (Österling et al. 2008, 2010, Strayer and Malcom 2012). This stage can become a serious bottleneck for freshwater mussel recruitment. In addition, my results showed that recruitment of *M. togakushiensis* can be determined not simply by a linear effect of independently acting factors but also by an interacting function of juvenile survival rate and the potential number of juvenile supplies, suggesting that higher values of both are necessary for the recruitment. My results provide important perspectives for future conservation measures of freshwater mussels worldwide. For example, when trying to recover the natural recruitment of freshwater mussels, it is recommended for managers to pay close attention to habitat quality for the post-parasitic juveniles. If the habitat quality is low, juveniles could not survive even in the habitat with the high potential number of juvenile supplies. In addition, such efforts can be most effective when done together with efforts that maintain a high level of juvenile supplies. The previous cases that didn't fully succeed to reestablish the recruitment have focused on a part of the life histories of freshwater mussel species (e.g. Galbraith et al. 2018). The reasons for partially unsuccessful efforts might be associated with the possibility that mussel recruitment was impaired at not only the targeted stages but also other life-history stages.

The results in the thesis imply that there are extremely high possibilities that future local extinction or dramatic population shrinkage of *M. togakushiensis* because the study demonstrated the relationship between recruitment, juvenile survival rate, and human activities. The results can be partially explainable from the concept "extinction debt" and are in line with a previously proposed time-lagged extinction of long-lived freshwater mussels in the non-fragmented rivers (e.g. Poole and Downing 2004). Importantly, it is acknowledged that the current findings are based on assessments of a part of river systems with the occurrence of this species and examined mussel status over only several years much shorter than their longevity. It needs to be reminded that the results and management implications need to be discussed within these limitations.

Toward the conservation of *M. togakushiensis* in eastern Hokkaido

The results in the thesis suggested that the proportion of juveniles of *M. togakushiensis* becomes high in the rivers with high juvenile survival rates and glochidia density. Therefore, both factors should be enhanced to recover the natural recruitment of *M. togakushiensis*. According to the estimations of the models constructed in Chapter 5, at least > 0.5 of the juvenile survival rate and 41 individuals/m^2 of glochidia density are required to maintain viable and functional populations (those with the proportion of juveniles being > 0.2 in populations). These values can be important criteria for the future conservation of the target species in the study region. The results also showed that juveniles cannot survive under low habitat quality even if sufficient glochidia density is maintained. Therefore, the managers need to place first priority in conservation efforts on the improvement of habitat quality. The juvenile survival rate is closely related to excessive nutrients and fine inorganic sediment. Thus, the reduction of nutrient inputs and fine sediment load can be an effective management option in efforts of habitat quality improvement. It needs to be stressed that threshold levels of environmental variables, 33.5 mS/m of EC and 4.2 mg/L of fine inorganic sediment, in surface water should not be exceeded to maintain juvenile survival rates of > 0.5 based on the estimations obtained from the models in Chapter 5.

The nutrient concentration (i.e. EC) was positively related to the agricultural land and urban cover ratios in the study catchments. The excessive nutrient inputs might be mainly derived from livestock wastes and fertilizers from agricultural land, and domestic and industrial sewages from the urban areas. To reduce the nutrient inputs into rivers, several management actions might be effective. First, wetlands and vegetated buffer strips may be effective to capture nutrients before these reaches stream (Weller et al. 2011). Actually, EC of river water had a negative relationship with wetland cover in the catchments in the study region (Fig. 6-1). Therefore, this management may be effective also in the study region to reduce the nutrient inputs into the rivers. Second, reducing the use of fertilizers and restricting their use in areas with tight hydrologic connections to streams are also important strategies to reduce the inflow of nutrients. Third, the legislation of the management of the composts derived from livestock waste may be also important to prevent the excessive inflow of nutrients. Japanese national row regulates the use and storage of livestock

wastes. For example, farmers are prohibited from piling up the livestock wastes in the open not to be flashed out to rivers directly (Ministry of Agriculture Forestry and Fisheries 2011). On the other hand, the use and storage of composts made by livestock wastes are not regulated by national row. The study area is mainly composed of three towns: Akkeshi, Bekkai, and Nakashibetsu Towns. Among three towns, only Bekkai Town has local legislation for the use and storage of composts made by livestock waste. The local legislation prohibits the improper use and storage of composts by specifying appropriate details such as those in terms of the timing of composting and storage places. Enacting such ordinance to control livestock wastes including composts might be also important in the other two towns. Forth, increasing the percentage of sewered population may be effective to reduce the nutrients inputs into rivers from urban. The latest reports showed that the percentage of sewered population (including establishing the septic tank) of Bekkai, Akkeshi, Nakashibetu Towns are now 86%, 80%, and 93%, respectively (Hokkaido 2019). The remaining populations drain domestic/industrial wastewater directly into the environment without effective treatments. To reduce pollution effects of these cases possibly on rivers, establishing the drainages and wastewater treatment plants or septic tanks are important. In addition, although the septic tank is known as an effective tool for the treatment of wastewater at a personal level, this needs periodic cleaning to maintain its functionality properly. The legislation that regulates personal management is also necessary to properly treat wastewater from urban areas.

In addition to the excessive nutrient inputs, fine inorganic sediment load negatively affected the recruitment of *M. togakushiensis* interactively with a nutrient concentration in the study region. Therefore, the reduction of the fine sediment load is necessary for recovering the natural recruitment of the target species. To reduce the fine sediment load in the rivers, determination of the source of it is imperative. However, the present study was unable to determine the source of fine sediment. The most suspicious source of fine sediments is agricultural land because of the high covering area ratio in the catchments, but the clear relationship between fine sediment of inorganic and agricultural land in the catchments was not found. This is explainable by the source of fine sediment being point sources rather than diffuse sources. Large amounts of fine sediments could be supplied to river water by local events in the catchment such as landslides, riverbank erosion, and deforestation (Wood and Armitage 1997). The undetectability of my study in actual sources of

sediment is assumingly associated partially with the limitation of the quantitative approach in land-use analyses. GIS-based information used in the analyses was not available in such a way that analyses with a spatial resolution high enough to examine or detect the effects of point sources on water quality were possible. Furthermore, among the study rivers, fine inorganic sediment in Rivers *s* and *r* were relatively abundant although agricultural land covers in the catchments were zero or low. At the time of the sampling occasion in 2018, the deforestation and management of woodland paths were locally conducted and observed in the two catchments. These human activities might potentially increase the fine sediment load in the rivers, affecting negatively mussel populations. However, GIS-based information used in the analyses was not sufficiently updated with a high level of temporal resolution to include such relatively recent changes in landscape characteristics. These spatial, as well as temporal mismatches between field conditions and available analytical data might have made it difficult to determine the relationships between land-use and fine sediment load. Determining the source of fine sediment is an important future challenge for recovering the natural recruitment of *M. togakushiensis* in the study region and the availability of better geospatial data might be one of the key technical challenges.

My results also showed that sufficient potential number of juvenile supplies (i.e. glochidia density) is necessary for recruitment of *M. togakushiensis*, together with a high survival rate of juveniles. Therefore, enhancing the glochidia density is an effective and important conservation measure to increase recruitment in the rivers where the potential number of juvenile supplies is low or lacking. This conservation measure may be more effective in the rivers where the juvenile survival rate is high but glochidia density is low (e.g. Rivers *f*, *g*, and *k*).

Glochidia density is a function of host fish density and mean glochidia load on host fish individuals. Therefore, increasing host density can be also effective for enhancing the glochidia density. Large wood generally plays important roles in stream hydraulics, sediment storage, and channel morphology (Bilby and Ward 1989, Nakamura et al. 1997), which are major determinants of fish habitat quantity and quality. The installation of large wood can provide habitats for fish by providing cover habitats and enhancing the habitat heterogeneities such as higher variations of water depth, current velocity, and the size of river-bed sediments, and can enhance fish density (Inoue and Nakano 1998, Nagayama et al. 2009). Therefore, the installation of large wood may be

also effective for increasing host fish density in the rivers from study regions. The installation of large wood in the river reach where *M. togakushiensis* is abundant. It is also strongly recommended that riparian forests as a source of large wood would not be disturbed or removed to maintain natural supplies of large wood.

Increasing the mean glochidia load is also effective for enhancing the potential number of juvenile supplies together with increasing host density. The (re-)introduction of host fish can increase host fish density while this needs to be considered by paying careful attention to the possibility of disruptions of genetic structures of fish. If this approach is acceptable, in particular, (re-)introduction during the reproductive period of *M. togakushiensis* can be more effective to increase host fish density and glochidia load on host fish individuals. In addition, artificial infection of glochidia larvae on host fish may be more effective for enhancing the glochidia load on fish individuals because a larger number of glochidia can be attached more reliably. This artificial propagation might be also an important conservation measure for recovering recruitment of *M. togakushiensis* in the future.

The management at the catchment scale, habitat improvement of host fish, and (re-)introduction of host fish and artificial propagation like above would recover and enhance the recruitment of *M. togakushiensis*. But, the reasons for the spatial variation of the fine inorganic sediment should be determined. These valuable findings from this thesis and implementing proposed conservation measures could contribute to maintaining the functional integrity of freshwater ecosystems in the study region by recovering freshwater mussel recruitment. The long-term monitoring of the study populations and changes of key-environmental factors such as land-use and availabilities of host fish should be continued to better predict future changes in population dynamics. Furthermore, locating other remaining habitats of *M. togakushiensis* and monitoring them is also recommended.

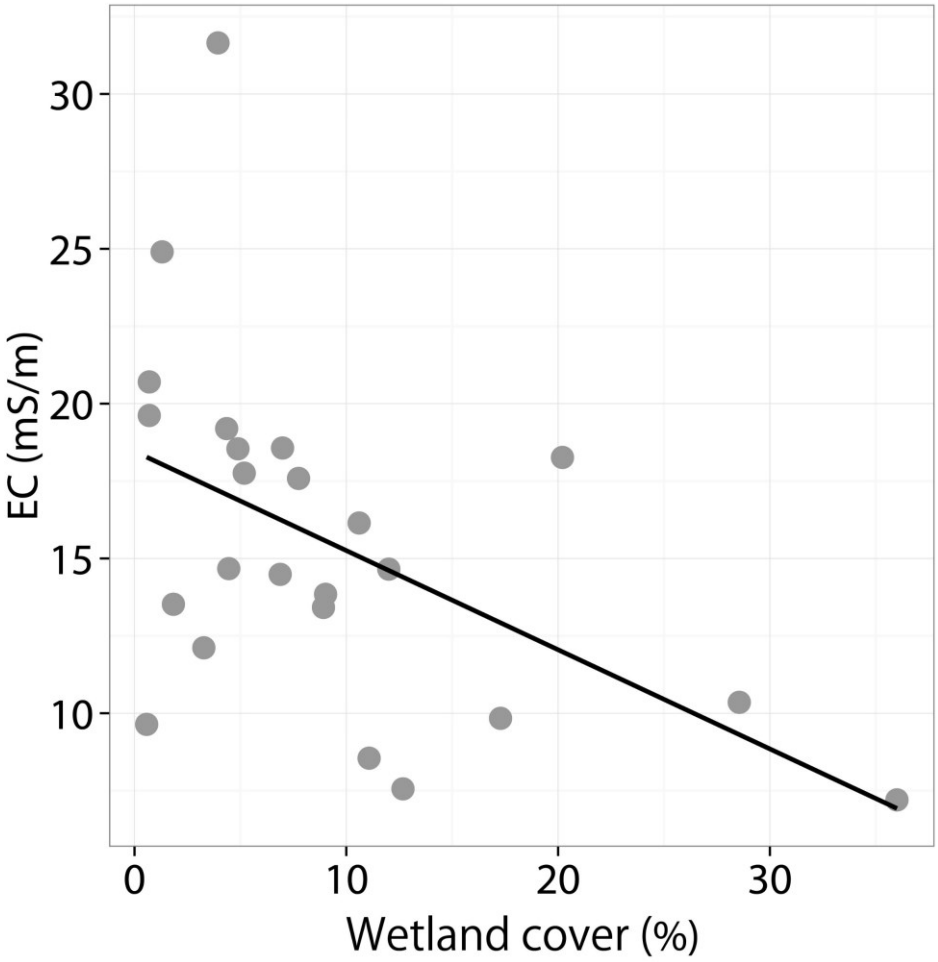


Fig. 6-1 Relationships between electrical conductivity (EC, mS/m) and wetland cover (%) in the catchments (a) in 24 study rivers targeted in Chapter 4 (including 11 study rivers targeted in Chapter 5). The black lines were drawn using the regression in the generalized linear models (GLM) with a Gaussian error distribution

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