The ecology and genetic structure of Palaemon paucidens populations with alternative life histories in Lake Biwa, Japan

鄔, 倩倩

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博　士　論　文

The ecology and genetic structure of *Palaemon paucidens* populations with alternative life histories in Lake Biwa, Japan

琵琶湖産スジエビの生活史多型に関した生態的特性及び遺伝的構造の解明

神戸大学大学院人間発達環境学研究科
人間環境学専攻　環境基礎論講座
学籍番号・氏名　142D841D　邬　倩倩

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Chapter 1. General introduction

The term ‘life history’ refers to an organism’s life cycle, which extends from birth and growth to reproduction and death. Ricklefs & Wikelski (2002) reported that life history is commonly defined as a set of evolving strategies, including behavioral, physiological, and anatomical adaptations. Such strategies directly influence an organism’s survival and reproductive success. It can be observed that organisms exhibit different behaviors during distinct developmental stages of their life history (Northcote 1978). Many properties of animal behavior are the result of natural selection. Animals evolved their behaviors to survive and reproduce better, and possess morphological characteristics suitable for their habitat environments (Lewontin, 1970; Williams, 1974).

However, not all individual members of a group exhibit the same life history or identical behavior (Sakei *et al.*, 1999; Gross, 1996), as has been observed in certain species of reptiles (Hatase *et al.*, 2004), birds (Berthold, 1991; Rolshausen *et al.*, 2009), fish (Bernatchez & Dodson, 1990; Foote *et al.*, 1989), and insects (Danks, 1994). Behavioral ecologists have confirmed the relationship between

genes and behavior (Fitzpatrick *et al.*, 2005; Bell & Robinson, 2011; Zayed & Robinson, 2012). The genotype is expressed as a trait of the organism, including the form, structure, behavior and physiological properties of the organism. However, individuals with the same genetic sequence, or even the same individual, can sometimes exhibit different behaviors.

Tinbergen (1963) pointed out that studying animal behavior is crucial in understanding "why behaviors are beneficial to survival and reproduction, and what kind of behavior will evolve?". In addition, studying polymorphic behaviors and life histories together offers a prime opportunity to elucidate the reason for which different phenomena are able to simultaneously occur within a single population (Lundberg, 1988; Kaitala *et al.*, 1993). Therefore, by studying animals' behavior, not only a more profound understanding of the species' current ecology and characteristics can be obtained, but the meaning of behavior in the evolution process is discerned. Therefore, the understanding of a given species in various aspects can be facilitated.

In this research, the target species is *Palaemon paucidens* (Crustacea, Decapoda, Palaemonidae). Based on its migration patterns, *P. paucidens* populations are classified into diadromous and landlocked populations which can be respectively divided into A-type and B-type as well according to the genetic differences between them (Chow & Fujio, 1985). It has been reported that the landlocked populations inhabit lakes and rivers during all their lives, while the diadromous populations are in the brackish water around the estuary or in the sea during the pelagic period and then return to the river (Chow *et al*., 1988). It is worth noticing that the population inhabiting Lake Biwa is considered landlocked which has non-migratory genetic characteristics (Kawane *et al*., 2014). It is not rare to see crustacean migration (i.e. *Portunus trituberculatus*, *Penaeus chinensis*; Liu *et al*., 2009; Meng *et al*., 2009), however the species inhabiting Lake Biwa shows an interesting ecology. Previous researches have reported the species has the characteristic of a seasonal deep-shallow migration in Lake Biwa despite it is genetically identified as A-type, which shows generally non-migratory behavior

(Nishino, 2008; Kawane *et al*., 2014). Such phenomenon about seasonal deep-shallow migration of this species not being seen in other crustaceans in freshwater lake. Most of the *P. paucidens* have been reported as seasonally migrating. They stay in the shallow water due to reproduction purposes during late spring and summer. From autumn, most of individuals migrate from the shallow water to the bottom of the lake to spend the winter (Harada, 1966; Nishino, 1983). It was reported some individuals (non-migratory individuals) overwinter in the shallow water (Harada, 1966). However, knowledge about these individuals is limited. The non-migratory individuals which stay in the shallow water during the winter have been estimated less than <1% of total population (Biological Resource Research Team in Lake Biwa, 1966). These behaviors have been recognized as the life-history polymorphism of Lake Biwa.

This species is an important food source for fish (Harada, 1966; Narita, 2002), and also important for the people in Shiga Prefecture, as it is regarded as an indispensable raw material in local cuisine for large-scale festivals, weddings and

funerals (e.g., *ebimame*). And the goods containing this species as one of its ingredients are sold in convenience stores and highway rest areas. However, amount of resources of this species has shown a significant decline in comparison with late 1970s (Shiga Prefecture, 2009).

While the species exhibits such a distinctive way of life, little research has been conducted on its ecology and distribution in the lake (Harada, 1966; Nishino, 1983). Harada (1966) hypothesized that *P. paucidens* migrates to the bottom of the lake during autumn in order to hibernate. However, if such movement was motivated by hibernation, then moving from the shore to a depth of 80m would not only be a waste of energy, but would also carry the risk of death from predators. Thus, it is worth further investigating the reason according to which this species is motivated to move to the bottom of the lake during winter. However, the knowledge of non-migratory *P. paucidens* individuals in Lake Biwa is limited and whether their existence as previously described remains uncertain. If non-migratory individuals do exist, it is possible that any differences

in their behavior could be caused by genetic variation or other reasons. To comprehend the reason why they exist this characteristic, the additional exploration to morphological characteristic between migratory and non-migratory individuals is needed.

Before discussing these issues, it is necessary to locate those shrimps that do not migrate, which, in such a huge lake, is a difficult and laborious task. In recent years, many studies have reported that environmental DNA (eDNA) analysis is successful in monitoring of the presence/absence of rare species, biodiversity, and abundance of populations in freshwater environments (Dejean *et al*., 2011; Thomsen *et al*., 2012; Fukumoto *et al*., 2015; Yamanaka & Minamoto, 2016a). Therefore, this study has focused on the following points. Firstly, to confirm the cause of the migration of *P. paucidens* to deep water, chemical experiments were completed to analyze changes in lipid and nucleic acid contents, as well as the stable isotope ratio before and after migration to deep water. Then, so as to demonstrate the presence of non-migratory populations, eDNA analysis was

applied to examine the distribution of overwintering populations in the shallow waters of Lake Biwa. Next, the genetic structure and ecological characteristics of migratory and non-migratory individuals were analyzed and their differences were discussed. Finally, the eDNA distribution of *P. paucidens* (migratory and non-migratory individuals) in Lake Biwa was tracked over a year, and considered whether the habitat preferences of *P. paucidens* are related to shoreline landscape types. In this study, through these four examinations, a new knowledge of the life history and the temporal and spatial distribution of this species in Lake Biwa was explored.

Chapter 2. The life history and seasonal migration of *Palaemon paucidens* in Lake Biwa

2.1 Introduction

Migration is defined as synchronized movements of individuals between distinct habitats that occurs at specific life stages (Lucas & Baras, 2002), and is common in several animals (Dingle & Drake, 2007): mammals (Avgar *et al*., 2014), birds (Berthold *et al*., 2003), amphibians (Sinsch, 2014), and fish (Secor, 2015).

Migration studies have described migration trajectories and examined environmental factors that may serve as proximate cues for migratory behavior. Additionally, research efforts have assessed the ultimate causal factors behind migration, i.e., those involved in the evolution of different migration strategies (Dodson, 1997). Migratory strategies are diverse in nature and are categorized by their purpose and function: i.e., (1) migration to a breeding site, (2) seasonal refuge from predators or adverse environmental conditions, and (3) migration to a feeding site (Northcote, 1978). These functions are not mutually exclusive and can co-occur in one species, as is seen in many bird species that seasonally migrate between overwintering and breeding sites (King *et al*., 1965). From an

evolutionary point of view, distinguishing these functions as causes or consequences will improve our understanding of the origin of migration. For example, food availability or predation risk may be characterized as driving forces of migration (Brönmark *et al.*, 2008), whereas decreased competition or changes in trophic dynamics may be classified as a consequence of migration (Brodersen *et al.*, 2008, 2011). Therefore, it is important to determine life history traits that are associated with migration to understand the evolution of migratory behavior.

The lacustrine shrimp *Palaemon paucidens* (Crustacea, Decapoda, Palaemonidae) is migratory in its life history. This species has the widest distribution among freshwater shrimps in the Far East Asia (Holthuis, 1950; Kim, 1977), and is found in southeastern Siberia, Sakhalin Island, the Japanese Archipelago, Korean Peninsula, and mainland China (Kim, 1976). In Japan, the distribution of this shrimp ranges widely from Amami Island to the northern limit of Hokkaido Island (Rathbun, 1902a; Kubo, 1942; Shokita, 1975; Suzuki *et al.*,

2015). This species occurs in a variety of freshwater habitats, from the mouth to the upper course of rivers, and from small ponds to large, deep lakes (Rathbun, 1902b; Kubo, 1942; Shokita, 1975). Based on their migration habits, populations of *P. paucidens* are generally classified as either diadromous or landlocked, which are reported to be genetically different (Chow *et al.*, 1988).

The landlocked population of *P. paucidens* in Lake Biwa in Japan exhibits seasonal migration within the lake (Harada, 1966; Nishino, 1983; Idomoto & Hatano, 2015). They are abundant in shallow lakeshore habitats from spring to summer, but are found in deep parts of the lake (about 90 m in depth) from autumn to winter (Harada, 1966; Nishino, 1983; Idomoto & Hatano, 2015). Recent spatiotemporal investigation on the distribution of *P. paucidens* showed that some individuals began migrating from the end of August, and that most individuals were distributed throughout the deeper layers in winter (Figure 2.1, modified from Idomoto & Hatano, 2015). Recent environmental DNA analysis

revealed that some individuals may overwinter in shallow waters of freshwater lagoons, which connect to the main lake (Chapter 3).

The ultimate cause and purpose of the migration of *P. paucidens* within Lake Biwa have not been elucidated. Harada (1966) hypothesized that migration to deep sites in winter was avoiding predation, because lakeshore aquatic plants wither in autumn and shrimps, therefore, lose their refuges from predators (i.e., the predation avoidance hypothesis). However, it is unclear whether migration to a depth of 90 m is necessary to reduce the risk of predation. It has also been hypothesized that *P. paucidens* migrates to deep sites for hibernation without feeding (i.e., the hibernation hypothesis; Harada, 1966), because such deep sites are believed to lack food for this species in winter. Although migration may be accompanied by energy loss and predation risk, these costs may be outweighed by benefits from other processes, such as foraging at deep sites. Based on studies of fish migration, Brönmark *et al.* (2008) suggested that seasonal migration may be adaptive in response to seasonal changes in predation mortality and growth rate,

wherein costs due to predation are minimized and benefits from foraging are maximized. However, little is known about the life history of *P. paucidens* in relation to its seasonal migration within Lake Biwa. *Palaemon paucidens* was hypothesized to forages at deeper sites in winter (i.e., the winter foraging hypothesis). The hibernation hypothesis and winter foraging hypothesis mutually exclusive wish to be highlighted, but that they are not mutually exclusive to the predation avoidance hypothesis.

The aim of the present study was to elucidate the life history traits of *P. paucidens* in relation to its seasonal migration within Lake Biwa, and to test the hibernation and winter foraging hypotheses. The former hypothesis predicts that the species hibernates in the deep parts of the lake in winter, ingests little nutrition, and has low physiological activity levels, while the latter hypothesis predicts that the species migrates to the deeper sites of the lake to forage from a specific nutritional source, and that it exhibits a certain level of physiological activity during this time. To test these hypotheses, the seasonal variation in

feeding ecology, individual growth, and reproductive conditions were investigated. The spatiotemporal patterns of body size, reproductive condition, lipid content, RNA/DNA ratio, and carbon and nitrogen isotope ratios were analyzed. Furthermore, the life history of the species and the importance of feeding in migratory and overwintering individuals were discussed.

2.2 Materials and Methods

2.2.1 Study area

Lake Biwa (35°01′ N, 136°00′ E, 669.23 km^2, Figure 2.2) is the largest freshwater lake in Japan, and the third oldest lake in the world. This lake harbors over 2400 species of animals, including 66 endemics (Kawanabe *et al*., 2012). The lake consists of two parts: the small, shallow south basin with an average depth of 3.5 m (Okuda & Kumagai, 1995), and the north basin which is the major part of the lake with a maximum depth of 104 m (Okamoto, 1984). The water temperature of the surface layer varies seasonally

from 7°C to 30°C, but hypolimnetic water temperature is stable at 6–7°C (Okamoto, 1992). Sampling was conducted in Shiotsu Bay, which is situated in the northernmost part of the north basin.

2.2.2 Sample collection

In the evaluation of seasonal migration and associated changes in body size and reproductive conditions, comparison between samples from different sites at the same time may be useful. To obtain spatio-temporal series of samples, a total of 11 collection trials were performed from shallow and deep sites in warm and cold seasons: November 14 (>-7 m and -40 m in depth) in 2014, and January 15 (>-7 m and -60 m), April 10 (>-7 m and -60 m), May 28 (>-7 m and -15 m), July 9 (-7 m), August 4 (-7 m), and September 16 (-7 m) in 2015 (Table 2.1). Collections were coordinated with the activity of a fishery, which cooperated in this study (Asahi Fishermen's Cooperative of Lake Biwa). A previous study reported that no or very few individuals were found at the offshore bottom in summer (Figure

2.1); as such, samples were not collected from deep sites in July, August, and September 2015. Trawl nets and basket traps were used to catch shrimps from deep (< -40 m) and shallow (> -15 m) sites, respectively. Sampling effort was similar across collection dates for each method (i.e., trawl net or basket trap), but sampling effort could not be controlled between the methods. Individuals with a carapace length less than 0.5 mm were excluded from my analysis, because these methods were unable to capture individuals smaller than 0.5 mm.

Shrimps were virtually absent in shallow sites in winter (Table 2.1), suggesting that they migrate from shallow sites to elsewhere (probably deep sites) in winter. Consequently, nine samples were not obtained from nine collection dates from November 14, 2014 to September 16, 2015 without spatial duplication on a collection date. The samples were immediately preserved with ice and transferred to the laboratory in Shiga University. Samples were stored in a freezer (at -20°C) until further analysis.

2.2.3 Body size and reproductive condition

To investigate the sex ratio and sexual difference in body size, 30 individuals were arbitrarily selected from each collection and sexed based on the appendix masculine on the second pleopod of endopod (Bruce, 1989). After thawing, the carapace was removed from the cephalothorax and the carapace length was measured to the nearest 0.1 mm using Vernier calipers, i.e., from the base of the eyestalk to the posterior margin.

To investigate the growth of *P. paucidens*, the body size of the other set of samples were measured. One hundred fifty individuals were arbitrary selected from every collection, resulting in a total of 1050 individuals throughout the study period. The gender of each individual was not distinguished because of large sample sizes. Instead, individuals that were carrying eggs and those with abdominal exoskeletons that remained deformed after spawning (Figure 2.3) (hereafter referred to as post-spawning female) were counted to investigate aspects of the species' reproductive ecology. It is unclear if the distinguishable

post-spawning exoskeleton condition is maintained after molting or if it is limited to a period before the next molt. Little is known about the molting schedule of *P. paucidens*.

2.2.4 Lipid content

Ingested nutritional substances are partly consumed and partly stored (e.g., lipids) for metabolism or growth (Boivin & Power, 1990). Individuals that ingest more nutritional substances are able to accumulate a higher amount of lipids in the body. On this basis, the total lipid content in *P. paucidens* samples were measured as a measure of the nutritional condition.

After thawing, individuals were weighed using a top loading electronic balance (Chyo, JS-110) to the nearest 0.0001 g. Specimens weighing ~5.5 g were analyzed: the total lipid content was extracted using methods described by Bligh and Dyer (1959) and the lipid content was measured using a conventional drying method according to Hasegawa (1993). The

whole body of shrimps was used in this analysis, but the eggs were removed if the females carried eggs. Arbitrarily chosen nine individuals were measured from each sample collection.

To explore the dependency of lipid content on reproductive condition, the lipid content was compared in three types of shrimps, that were female carrying eggs (excluding eggs), post-spawning females, and others (male or female before reproduction). Samples from July, August, and September in 2015 were used, because relatively large number of females carrying eggs were obtained in these months. In addition, the lipid content in the eggs removed from the females was also measured.

2.2.5 RNA/DNA ratio

The RNA/DNA ratio is an index of the gene expression activity of organisms and can be used as another measure of nutritional condition. The rationale for using the RNA concentration as a proxy for nutritional condition and growth rate

has been well developed (Buckley, 1984; Ferron & Leggett, 1994; Westerman & Holt, 1994; Bergeron, 1997; Buckley *et al.*, 2000). It is expected that well fed and fast-growing individuals exhibit higher RNA/DNA ratios than those that are starved (Wright & Martin, 1995; Hovenkamp, 1990; Hovenkamp & Witte, 1991).

After measuring the carapace length and removing external skeletons and intestines, the muscles of each sample individual were separated and stored in two experimental tubes for the extraction of RNA and DNA, following the methods described by Buckley (1979). Nine individuals were analyzed from each sample collection. RNA and DNA concentrations were calculated from their absorbance at 260 nm (Buckley, 1979). Samples from the September 16, 2015 collection were not analyzed because of a limited opportunity to conduct analyses.

2.2.6 Stable isotope ratio

To estimate the food source and trophic level of *P. paucidens* in Lake Biwa, the carbon and nitrogen stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) were analyzed, respectively (DeNiro & Epstein, 1978; DeNiro & Epstein, 1981; Minagawa & Wada, 1984).

After measuring carapace length, the muscle tissues of specimens (nine individuals per sample collection) were dissected out and dried at 60°C for 24 h. The samples were pulverized and immersed in 2:1 chloroform-methanol solution for 24 h to remove the lipids (Bligh & Dyer, 1959). Carbon and nitrogen stable isotope ratios were measured with a mass spectrometer (EA Conflo IV + delta V Plus; Thermo Fisher Scientific) at the Center for Ecological Research of Kyoto University. Isotopic notations of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) were expressed as per mil-deviation from the standards (atmospheric N_2 gas for nitrogen and PeeDee belemnite carbonate for carbon) as defined by the following equation:

$$\delta^{13}C \text{ or } \delta^{15}N = \frac{(Rsample - Rstandard)}{Rstandard} \times 1000\ (‰),$$

where R is $\delta^{15}N / \delta^{14}N$ or $\delta^{13}C / \delta^{12}C$. The analytical errors of $\delta^{13}C$ and $\delta^{15}N$ values were ± 0.1‰. Samples from the September 16, 2015 collection were not analyzed because of a limited opportunity to conduct analyses.

To estimate potential food sources, data was used from Yamada *et al*. (1998) and Narita (2002), i.e., the stable isotopic ratios of organisms (the amphipod *Jesogammarus annandalei* and oligochaetes) and organic matters (detritus and particulate organic matters [POMs]) from the shallow and deep sections of Lake Biwa, respectively. A general food chain study by DeNiro and Epstein (1978) reported that animal bulk $\delta^{13}C$ values are similar to those in their diet (<1‰ difference between the animal and its diet). Nitrogen isotopes increase by ~3.4‰ at each trophic level and can be used to determine trophic position (Minagawa & Wada, 1984; Vander Zanden & Rasmussen, 2001; Post, 2002). These criteria were used to estimate the potential food sources of *P. paucidens* in Lake Biwa.

2.2.7 Statistical analysis

Seasonal variations in body size and lipid content were examined using a one-way analysis of variance, with the collection date as the explanatory variable. Sexual difference in body size was examined using a general linear model with a normal distribution, with the collection date, sex and their interaction as the explanatory variables. Seasonal variation in the sex ratio (the proportion of females to total individuals) and the proportion of reproductive females to total individuals were examined using generalized linear models with a binomial distribution, in which the sex ratio or the proportion of reproductive females was used as the response variable, and collection date as the explanatory variable. The ratios of RNA/DNA, and carbon and nitrogen stable isotopes were analyzed by general linear models with a normal distribution, with the collection date, body size, and their interaction as the explanatory variables. Non-significant interactions were excluded from the final models. When the effect of collection date was significant, Tukey multiple comparison tests between collection dates

were performed. All analyses were performed using R version 3.3.2 (R Core Team, 2016).

2.3 Results

2.3.1 Body size and reproductive condition

Although the body size significantly varied among collection dates and the ranges of body size variation overlapped between the sexes, the females were significantly larger than males consistently across collections (GLM, sex, $F_{1, 208} = 50.27$, $P < 0.0001$; collection date, $F_{6, 208} = 53.19$, $P < 0.0001$), based on each of 30 individuals from 7 collection dates (Figure 2.4). The sex ratio did not differ among collections in deep sites in winter (Figure 2.5). In contrast, the sex ratio differed significantly among collections in summer: the proportion of the female was significantly lower in July (-7 m) and higher in September (-7 m),

indicating a gradual increase in the proportion of females in shallow sites in summer (Figure 2.5).

Based on each of 150 individuals from 7 collections, the carapace length varied between 0.5 mm (the lower limit of capture size) and 11 mm throughout the study period from November 2014 to April 2015 (Figure 2.5). Carapace length differed significantly between collection dates (ANOVA; $F_{6, 1043} = 41.17$, $P < 0.0001$). Mean carapace length increased significantly from November 2014 to January 2015 and decreased significantly in April 2015 in the deep site. In the shallow site, the mean carapace length was the smallest in collections from May 2015 and differed significantly from samples from other collection dates. The mean carapace length was seen to gradually and significantly increase until September 2015.

The proportion of reproductive females differed significantly (9–42%) between collection dates (GLM, $F_{2, 6} = 43.5$, $P < 0.0001$; Figure

2.4), and were significantly higher in January 2015, July 2015, and August 2015 than in April 2015 and May 2015. Individuals carrying eggs were found in shallow sites in August and September, and females with post-spawning exoskeleton conditions were found in both summer and winter.

2.3.1 Lipid content

Lipid content showed obvious seasonal variation (Figure 2.6A) and differed significantly between collection dates (ANOVA; $F_{6, 14} = 12.19$, $P < 0.001$). Lipid content peaked in January in the deep site and in May in the shallow site, with a significant decline in April in the deep site, indicating active feeding in winter as well as in the warm season.

The lipid content in post-spawning females was significantly higher than that in females carrying eggs and in other individuals (GLM, $F_{2, 22} = 30.62$, $P < 0.0001$; Figure 2.6B). Lipid content in eggs was higher than that in shrimp bodies.

2.3.2 RNA/DNA ratio

The RNA/DNA ratio differed significantly among collection dates and increased with increasing body size (GLM, collection date, $F_{6, 47} = 20.73$, $P < 0.0001$; body size, $F_{2, 47} = 95.17$, $P < 0.0001$) with a weak but significant interaction between collection date and body size ($F_{5, 47} = 2.71$, $P = 0.031$), but posthoc multiple comparisons did not detect significant differences between collection dates ($P > 0.05$). Variation in body size can involve sexual difference, because the male was significantly smaller than the female as shown above. Thus, the RNA/DNA ratio was relatively stable throughout the seasons after controlling the variation in body size and sex together.

For descriptive purposes, individuals were divided into three size categories: large (>6.0 mm), medium (3.0–6.0 mm), and small (<3.0 mm) (Figure 2.7). The RNA/DNA ratio tended to increase from April to August, especially in smaller individuals, suggesting a relatively high activity of gene expression in the young and growing stages and/or male individuals.

2.3.3 Stable isotope ratio

Variation in the carbon stable isotope ratio ($\delta^{13}C$) involved significant interaction between collection date and body size ($F_{5, 47} = 9.72$, $P < 0.0001$), indicating that temporal changes in food sources differed among growth stages and/or sexes. Post-hoc tests revealed that $\delta^{13}C$ values did not differ among body size categories in each of collection dates ($P > 0.05$) and differed across the collections (Figure 2.8A). This suggests that there was no consistent difference in food sources in relation to body size. Additionally, $\delta^{13}C$ values were significantly higher in July, August, and November than in January and May (Figure 2.8A), indicating that food sources differed between summer and winter.

The nitrogen stable isotope ratio ($\delta^{15}N$) did not differ between collection dates and was not associated with body size (collection date, $F_{6, 47} = 1.69$, $P = 0.144$; body size, $F_{2, 47} = 0.02$, $P = 0.903$, Figure 2.8B), suggesting that the trophic level remained unchanged throughout the year.

The C-N map of *P. paucidens* and its potential food sources are shown in Figure 2.8. The $\delta^{13}C$ and $\delta^{15}N$ values were -20.8‰ to -23.8‰ and 11.3‰ to 13.5‰ in winter, respectively, indicating that the food chain starts from detritus, as well as the amphipod *Jesogammarus annandalei* and Oligochaete (Figure 2.9). In summer, the $\delta^{13}C$ and $\delta^{15}N$ values of *P. paucidens* were -20.5‰ to -24.1‰ and 11.4‰ to 13.1‰, respectively (Figure 2.9), suggesting that this species may consume relatively small particulate organic matter (POM, 2.7–20.0 μm).

2.4 Discussion

Observed temporal changes in the body size, sex ratio and reproductive condition of *P. paucidens* in Lake Biwa offer novel insights into the life history of this species. The mean body size was the smallest in the shallow site in May, at which time the smallest proportion of reproductive female was observed. The body size was seen to gradually increase thereafter (in the shallow site) until September. In this period, the proportion of females (i.e., sex ratio) tended to decrease until

July, and then increased until September, and that of reproductive (egg-carrying) females increased from July to August but tended to decrease in September. These results suggest that young (small) shrimps joined the population in the shallow sites in spring and grew up there during summer. As the sex ratio changed during this period and females were larger than males, the observed change in body size may also be influenced by variation in the sex ratio, besides individual growth. Although it is unclear whether the observed variation in sex ratio is due to sex-related ecological difference or sampling error, increase in mean body size from July to September may partly reflect the increased ratio of females (47% to 97%, Figure 2.5). Relatively large individuals started to reproduce between May and July, with a peak between August and September; and that a portion of the post-spawning female population disappeared in August to September, probably due to die-offs after reproduction, which were indicated. During this long reproductive period from May to September, a female might reproduce multiple times, but little is known about multiple spawning in a single reproductive season in this

species. Relatively small individuals did not exhibit any indicators of spawning during this period. As the females were larger than the males in this species (see also Ogawa & Kakuda, 1988) and the females were relatively abundant in my samples (Figure 2.5), these small individuals were presumed to include males in addition to small females before reproduction. In November, both relatively large reproductive females and small individuals without indicators of spawning were found in the deep sites, where the body size only slightly increased and the proportion of post-spawning females remained unchanged until January. Then, interestingly, relatively large individuals were not observed in the deep sites until April, which may have been due to die-offs of these large individuals or they may have moved to sites that the samples were not collected from. A decrease in the proportion of large individuals was also observed in the shallow sites in May; however, it is difficult to discriminate whether the large individuals disappeared or the other a large number of small individuals joined, or both. These results suggest that small, non-reproductive individuals can overwinter and may

reproduce in their second summer; and that a small proportion of reproductive individuals can overwinter and may survive until the next summer. *Palaemon paucidens* in Lake Biwa may have a life-cycle that spans more than 1 year, despite previous studies having suggested that this species has an annual life-cycle (Harada, 1966; Nishino, 1978).

The present study provides novel findings regarding intraspecific variation in the adult lifespan of *P. paucidens*. The lifespan of this species has been reported to differ among regions in Japan. It is assumed to be a univoltine species with the die-off of females occurring after spawning in a pond in Nagano Prefecture (Okubo, 1961). In contrast, Handa and Araki (1930) found that a certain proportion of females survive for 2–3 years in Hokkaido. The findings of the present study support the latter case, wherein the adult lifespan of *P. paucidens* is longer than a year in Lake Biwa was found. Intraspecific variation in lifespan has also been observed in various kinds of fish. Some proportions of reproductive

trout survive after the first spawning and return to the sea, and then breed again in the following year (Kiso & Kosaka, 1994; Morita, 2001). In Lake Biwa, most individuals of the land-locked ayu-fish *Plecoglossus altivelis* exhibits a univoltine life-cycle and die-off after the first spawning; however, a portion of reproductive adults survive after the first spawning and reach sexual maturation in the second year (Matsuyama & Matsuura, 1984; Matsuyama, 1985). It is worth investigating if these same factors (or others) can explain the long lifespan of *P. paucidens* in Lake Biwa.

Previous studies hypothesized that *P. paucidens* migrate to deep waters to hibernate (i.e., the hibernation hypothesis; Harada, 1966). However, my findings do not support this hypothesis. The *P. paucidens* that collected in deep waters in winter having no hibernate was found and, instead, were observed to have been physiologically active: body lipid content was found to be the highest in January (i.e., increased over winter) (Figure 2.6A), the RNA/DNA ratio in winter was the same as observed in the warm season (Figure 2.7). Additionally, the carbon stable

isotope ratio indicated a difference in food sources between summer and winter (Figure 2.8B), suggesting that *P. paucidens* foraged in deep sites in winter. In this study, therefore, provides support for the winter foraging hypothesis. Although *P. paucidens* was found to forage in the deep sites in winter, growth rates appeared to be low during this period (November to January, Figure 2.5). This suggests that the nutrition gained in deep sites in winter was allocated for processes other than individual growth.

Prosser and Brown (1961) reported that mammals and birds store lipid in their body before overwintering. Fish species inhabiting cold and temperate regions also exhibit this trait (Brown, 1957). In contrast, the lipid content of *P. paucidens* increased during overwintering and being the highest in January, and then decreased until a second peak in lipid content occurred in May was observed. These lipid fluctuations corresponded to the onset of the growing and reproductive seasons (Figure 2.6). These results suggest that this species stores lipid for purposes other than

overwintering. Atsumi (1990) reported that the ovaries of *P. paucidens* began to grow from November, and that the egg-bearing females appeared from mid-March to April in a reservoir in Hyogo Prefecture, Japan. Although egg-bearing females were not found in winter to spring (but found post-spawning females; Figure 2.5), the temporal correspondence between the amount of stored lipid and ovary growth and egg production suggests that *P. paucidens* stored lipids during winter by foraging at deep sites (resulting in the highest amount of lipid in the body in January), and then used these reserves for reproductive purposes (resulting in a decrease in stored lipid in April). This is congruent with the results of the study that egg-carrying females stored very low levels of lipid in their bodies, while eggs were highly lipid-rich (Figure 2.6B). The second peak of lipid content in May might be attributed to subsequent foraging in warm shallow sites. Thus, the purpose of migration and winter foraging by this species in deep sites may be to gain nutrients for reproductive purposes. In addition, foraging in deep sites may also reduce the risk of starvation and compensate for the energy costs of the

migration behavior. The observed fluctuation in lipid content could also be explained by fluctuation in the sex ratio and/or composition of reproductive individuals, because discriminate individual sex and reproductive conditions were not discriminated in the analysis of seasonal change in lipid contents (Figure 2.6A). The lipid content was significantly higher in post-spawning females than in other individuals, but the proportion of female was unchanged, or rather decreased, during this period (i.e., January to May; Figure 2.5). Thus, the observed peaks in lipid content are unlikely to be attributed to the increase in the number of females.

In conclusion, a novel aspect of the life history of *P. paucidens* with regard to its seasonal migration in Lake Biwa was revealed. The hibernation hypothesis was opposed and the winter foraging hypothesis was supported, in that *P. paucidens* migrated to feeding sites in winter probably to obtain resources for reproductive purposes. In this study, the seasonal migration-predator avoidance hypothesis was not examined and further investigations are needed in this regard.

The present study provides a good example for understanding the life history traits and ecology of the seasonal migration of animals.

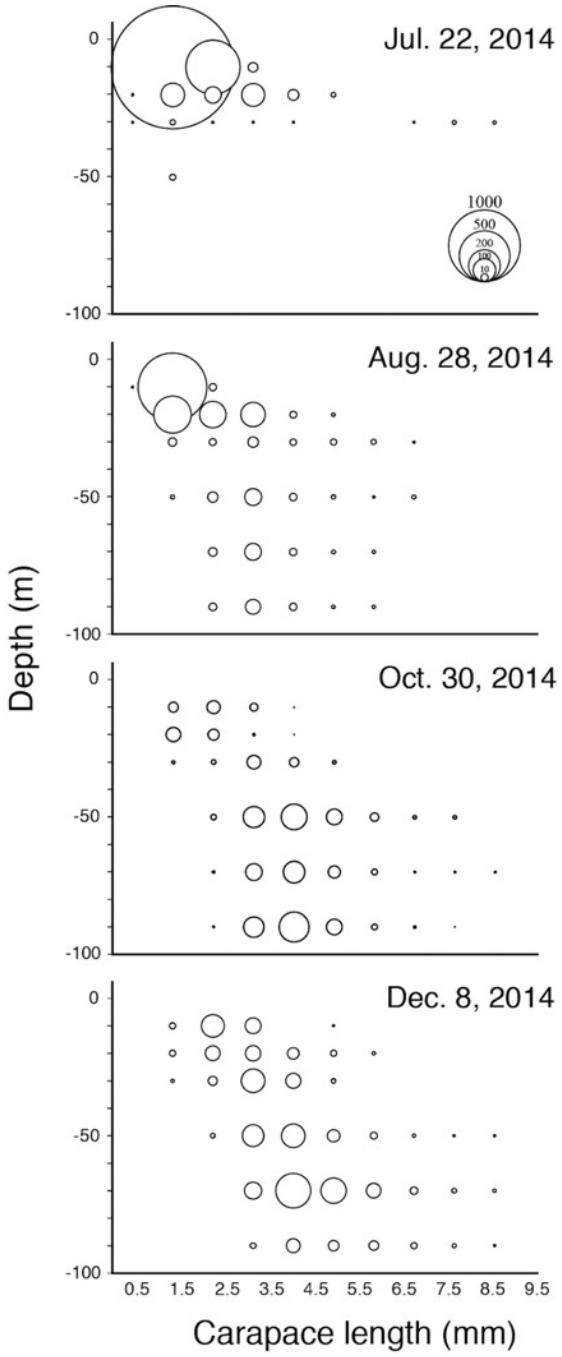

Figure 2.1. The spatiotemporal distribution of *Palaemon paucidens* in the north basin of Lake Biwa, indicating seasonal migration between shallow and deep sites. A trawl net (aperture size, 50 cm × 25 cm) was dragged for 70 m in each site (Idomoto & Hatano 2015). Circle size indicates the number of captured individuals. Plots were modified from Idomoto & Hatano (2015).

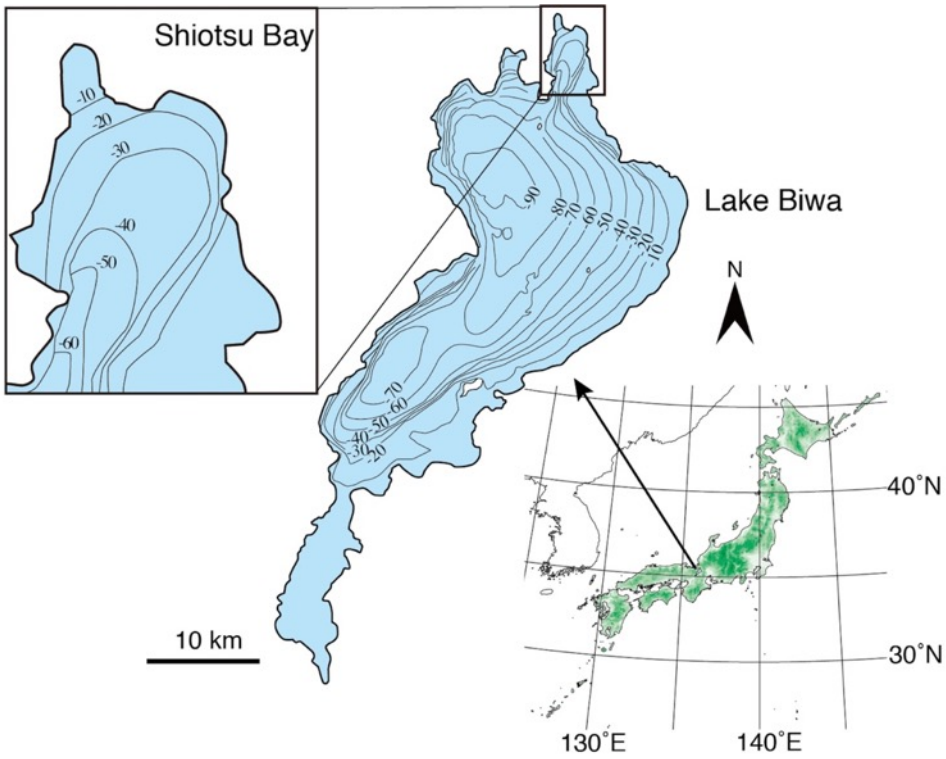

Figure 2.2. Map of the study site, Shiotsu Bay, located in the North Basin of Lake Biwa, Japan. The map of Lake Biwa was modified from Ikeda (2018).

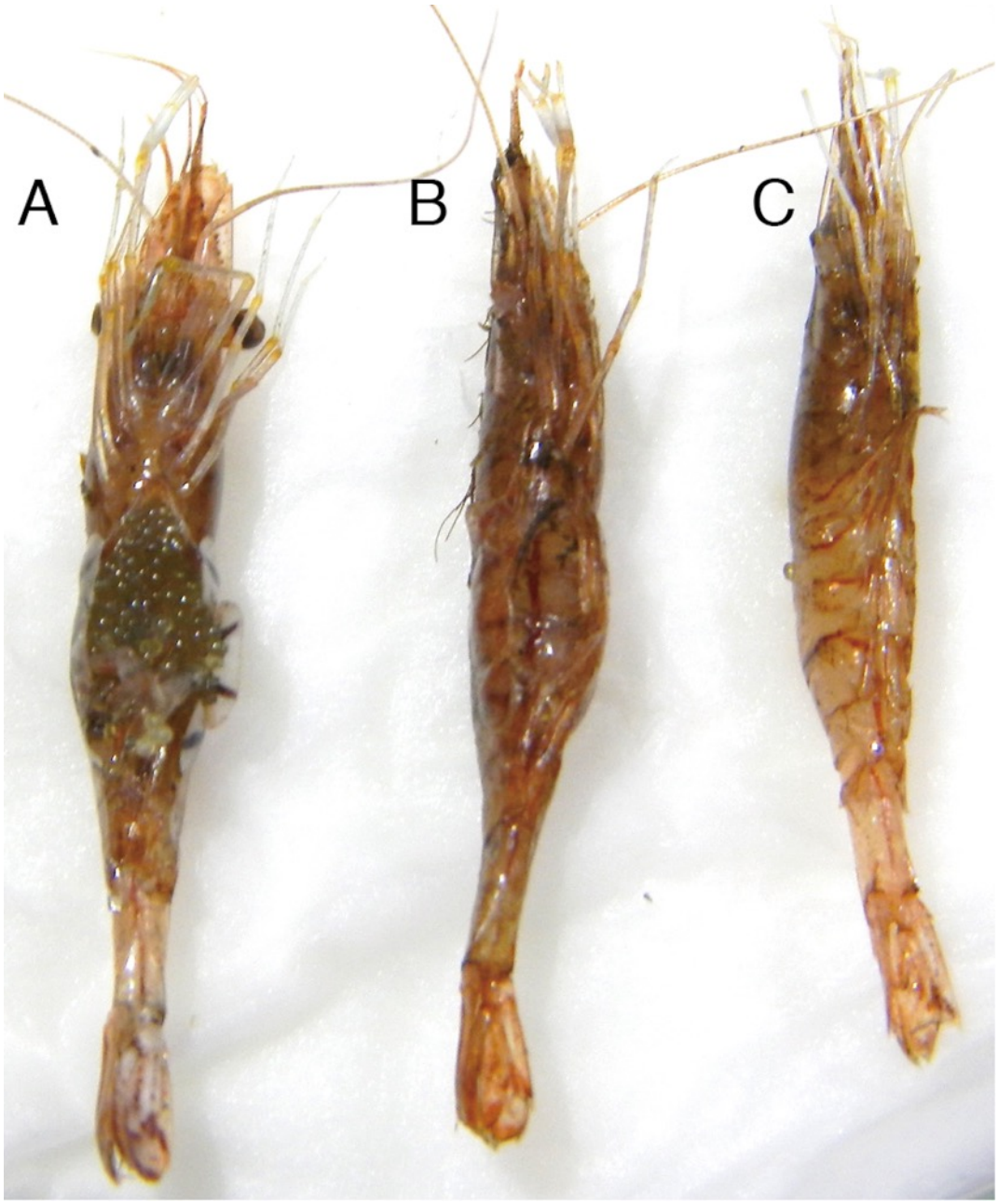

Figure 2.3. Variation in the reproductive condition of *Palaemon paucidens*. A, female carrying eggs; B, female with post-spawning exoskeleton condition (i.e., remained deformed); C, non-reproductive female or male.

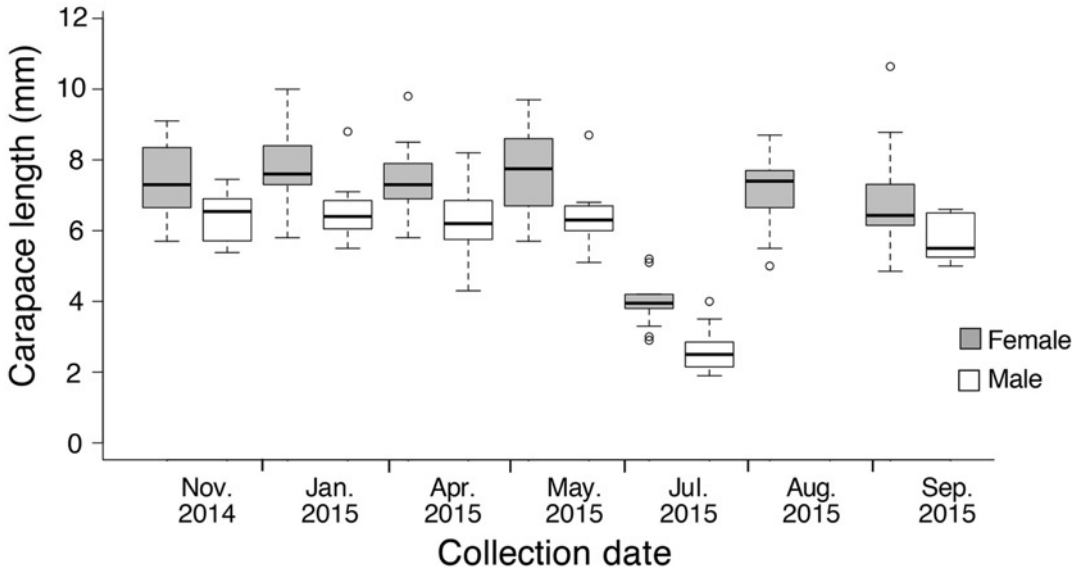

Figure 2.4. Temporal and sexual variation in the carapace length in *Palaemon paucidens*. Grey and white denote the female and male, respectively.

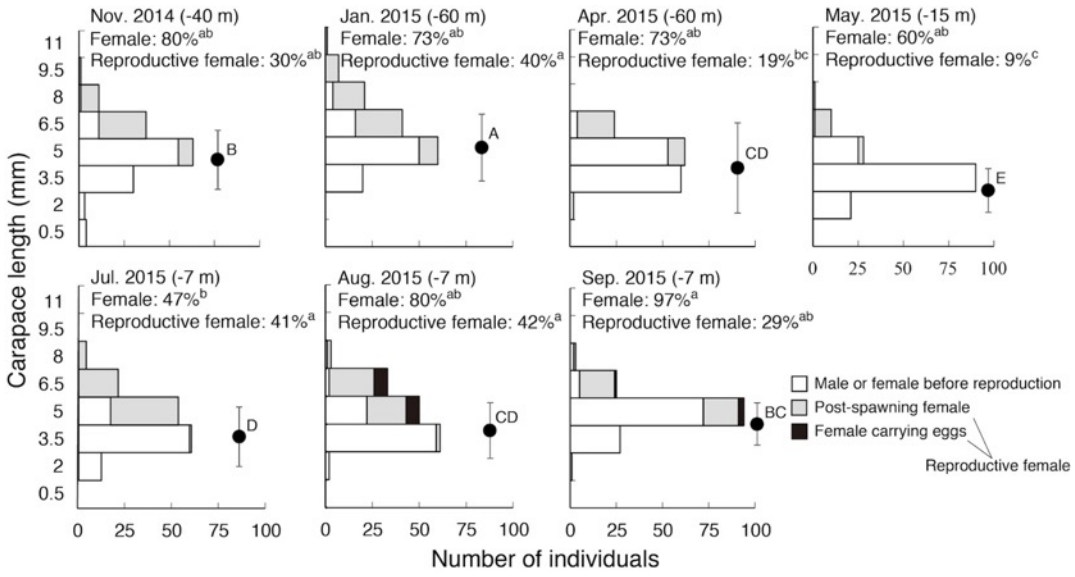

Figure 2.5. The seasonal change in the carapace length distribution, sex ratio and reproductive condition of *Palaemon paucidens* in Lake Biwa. Percentages indicate sex ratio (the proportion of females) and the proportion of reproductive females. Dots and bars indicate the means and standard deviations of carapace length. Lowercase letters indicate statistically significant differences in the sex ratio and the proportion of reproductive females. Uppercase letter indicates statistically significant differences in the mean carapace length.

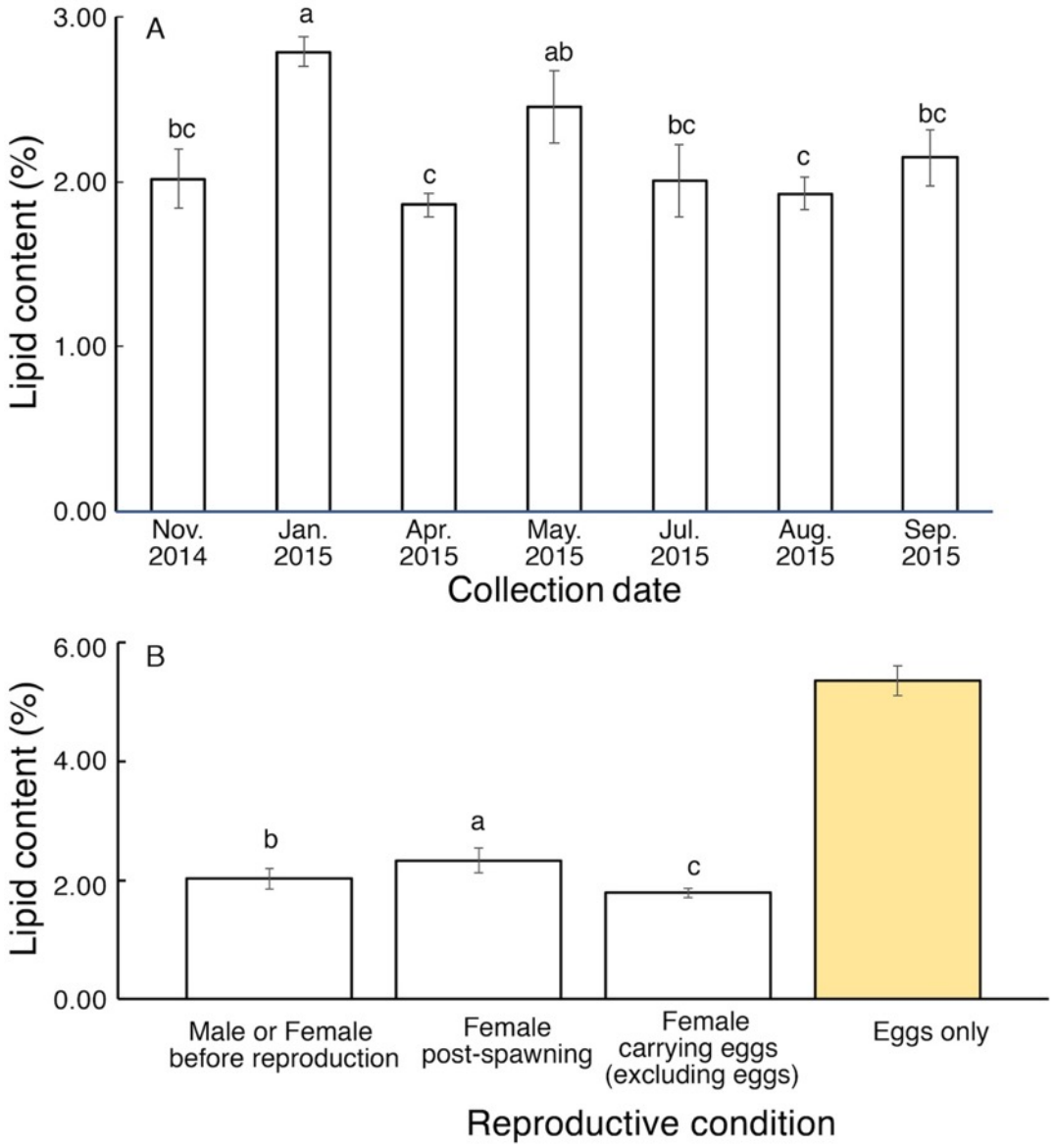

Figure 2.6. The lipid content of *Palaemon paucidens*. (A) Temporal variation in the lipid content of *P. paucidens* (n = 3 for each sample collection). (B) Different reproductive conditions of *P. paucidens* (n = 9 for each reproductive condition; n = 3 for eggs). Means and standard deviations are shown. Different letters indicate statistically significant differences.

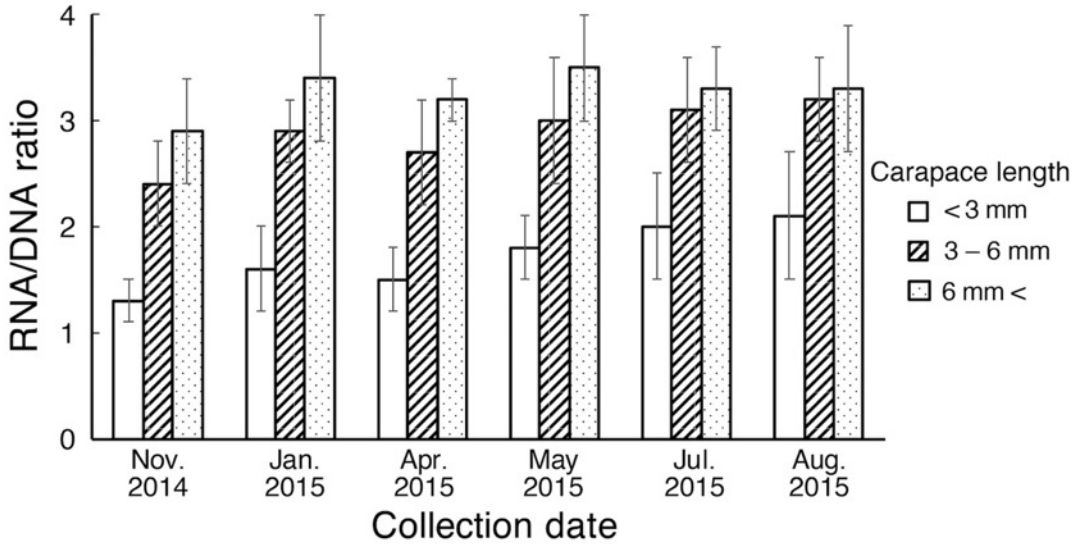

Figure 2.7. Temporal variation in the RNA/DNA ratio of *Palaemon paucidens*. Means and standard deviations are shown (n = 9 for each sample collection). RNA/DNA ratio was positively associated with carapace length, but did not differ significantly between collection dates.

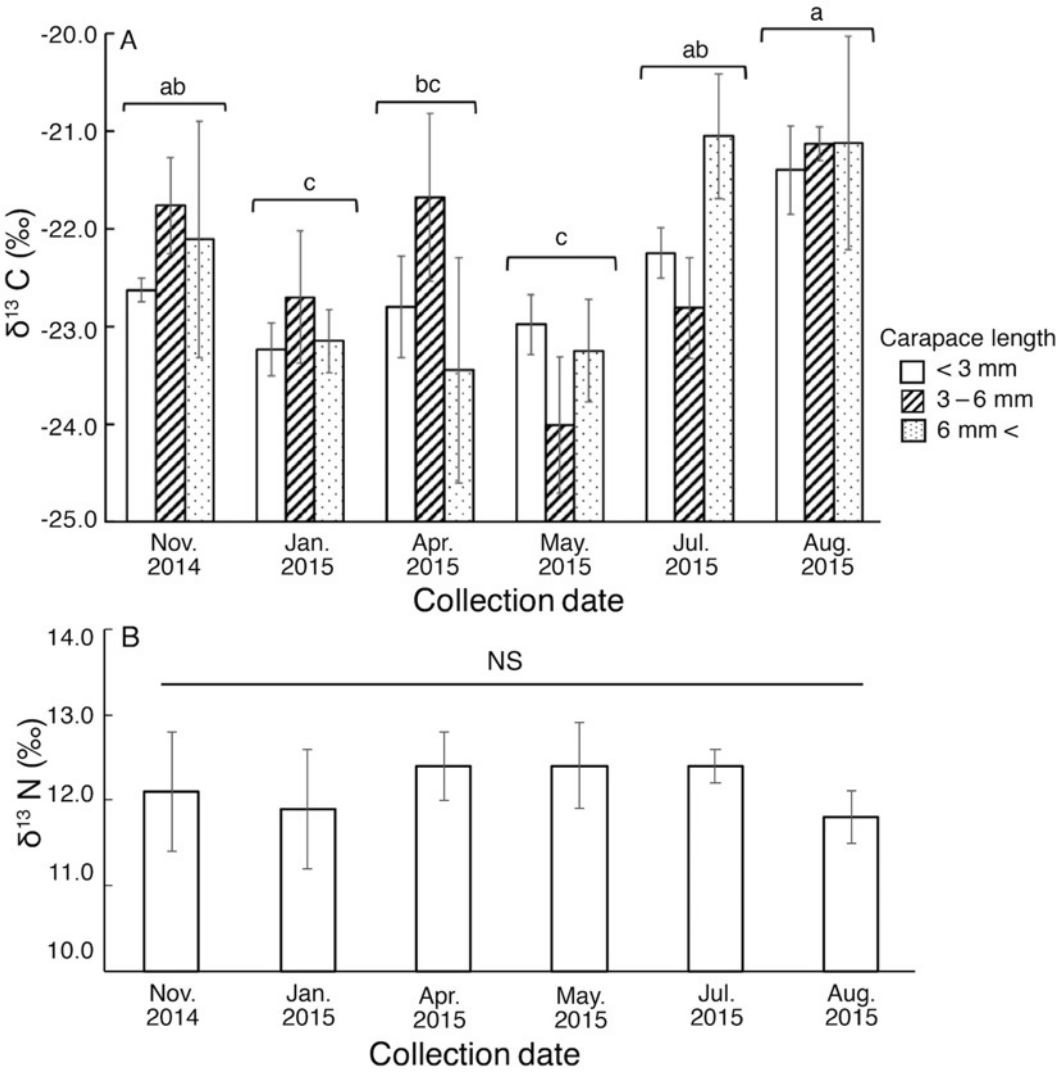

Figure 2.8. Temporal variation in (A) carbon stable isotope ratio and (B) nitrogen stable isotope ratio of *Palaemon paucidens*. Means and standard deviations are shown (n = 9 for each sample collection). Different letters indicate statistically significant differences. There was no significant difference in the nitrogen stable isotope ratio between collection dates.

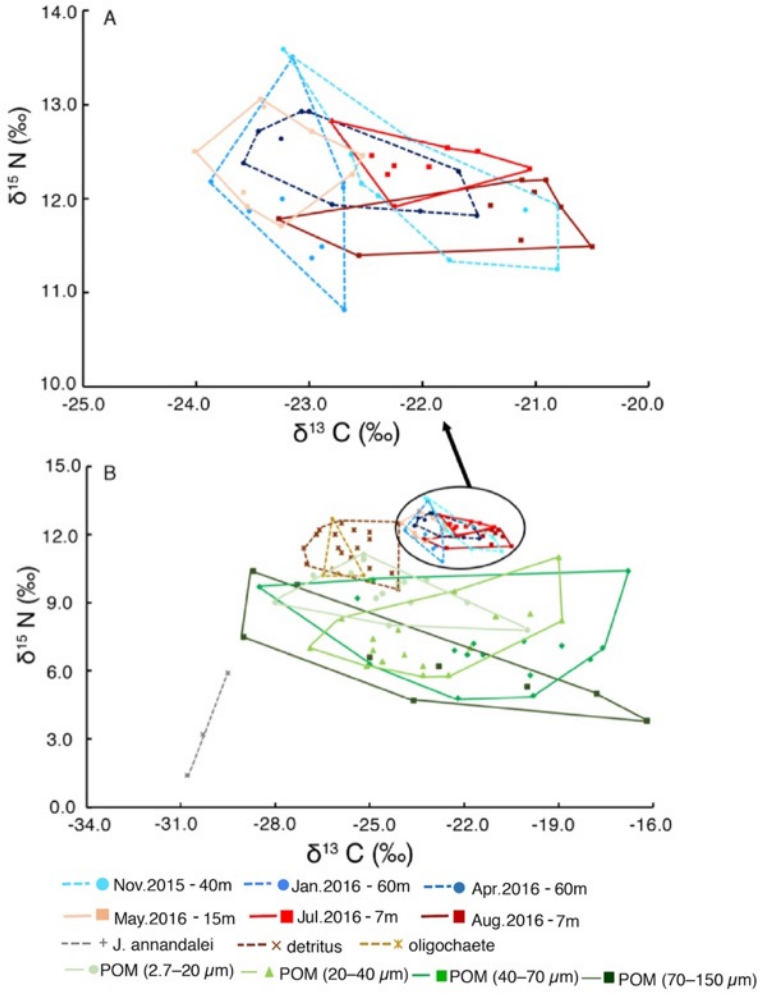

Figure 2.9. (A) The C-N maps of *Palaemon paucidens* and (B) its possible food sources in Lake Biwa. The range from sky blue to deep blue within dotted lines indicate the shrimps that were collected in deep sites in winter; the range from flesh color to dark red within solid lines indicate the shrimps that were collected in shallow sites from spring to summer. Data of *Jesogammarus annandalei*, detritus and Oligochaete in deep sites (grey, brown, dark goldenrod; dotted line) are obtained from Narita (2002). Data of particulate organic matter (POM) (2.7–20.0 μm), POM (20–40 μm), POM (40–70 μm) and POM (70–150 μm) in shallow sites (the range from light green to dark green; solid line) are obtained from Yamada *et al.* (1998).

Table 2.1. Number of collected *P. paucidens* individuals and the information of depth and survey tool on each collection date.

Collection Date	**Depth (m)**	**Survey tool**	**N**
14/11/2014	-40	Trawl net	<3000
	> -7	Basket traps	0
15/01/2015	-60	Trawl net	<3000
	> -7	Basket traps	0
10/04/2015	-60	Trawl net	<3000
	> -7	Basket traps	0
28/05/2015	-15	Basket traps	<3000
	> -7	Basket traps	0
09/07/2015	-7	Basket traps	<3000
04/08/2015	-7	Basket traps	<3000
16/09/2015	-7	Basket traps	<3000

Chapter 3. Using environmental DNA to demonstrate the presence of non-migratory individuals of *Palaemon paucidens* overwintering in shallow shores of Lake Biwa

3.1 Introduction

Freshwater habitats, such as lakes, rivers, and wetlands, are crucial for life on Earth. Freshwater accounts for only 0.01% of the world's water and cover ~0.8% of the Earth's surface (Dudgeon *et al*., 2006), but provides habitat for almost 10% of known species (Balian *et al*., 2008). Several investigators have reported that species diversity in freshwater habitats is far lower than in terrestrial habitats because of human activities (Collen *et al*., 2014; Cumberlidge *et al*., 2009). Thus, to protect freshwater biodiversity and environments, basic information on the distributions of species, populations, and intraspecific variants is indispensably required.

The study was focused on *Palaemon paucidens*, which belongs to the family Palaemonidae and is widely distributed over East Asia (Kim 1977; Chow & Fujio, 1985) where it inhabits southeastern Siberia, Sakhalin Island, the Japanese Archipelago, Korea, and mainland China (Kim, 1976). In Japan, the distribution of this shrimp species ranges from the southern limit of Yaku Island to the

northern limit of Hokkaido Island (Rathbun, 1902a, b; Kubo, 1942; Shokita, 1975). It appears in a variety of freshwater habitats, from small ponds to large deep lakes and from upstream to the river mouth (Rathbun, 1902a, b; Kubo, 1942; Shokita, 1975). Based on migration patterns, *P. paucidens* generally is classified into diadromous and landlocked populations (Chow & Fujio, 1985). The population inhabiting Lake Biwa is considered landlocked (Kawane *et al*., 2014), and previous investigators reported that individuals belonging to this population lived in deep waters from autumn to winter and in shallow waters from spring to summer (Harada, 1966; Nishino, 1983). However, some individuals have been reported as overwintering in the shallow waters of Lake Biwa. A previous reporter estimated that the proportion of such non-migratory individuals was <1% (Biological Resource Research Team in Lake Biwa 1966). However, knowledge on non-migratory *P. paucidens* individuals in Lake Biwa is limited, and whether they are indeed non-migratory has not been established. Therefore, knowing the

precise location of the habitat of non-migratory *P. paucidens* individuals in winter would help my understanding of the peculiar life history of this species.

Conventional methods for shrimp surveys using traditional sampling gear (i.e., shrimp cage, fyke netting, electric bait trapping) have low sensitivity and present detection biases in the census of shrimp populations. In contrast, environmental DNA (eDNA) analysis, a method that has been developing rapidly, may provide quantitative information on the abundance of shrimp populations and on species' presence/absence because it is based on the genetic materials released by organisms into the environment through excreted mucus, feces, or other wastes (Ficetola *et al.*, 2008). Investigators who used eDNA analysis have reported successful monitoring of the presence/absence of rare species, biodiversity, and abundance of populations in freshwater environments (Dejean *et al.*, 2011; Thomsen *et al.*, 2012; Fukumoto *et al.*, 2015; Yamanaka & Minamoto, 2016a). Several researchers have focused on the eDNA of fish and amphibians, but studies of crustacean eDNA are still scarce (but see Deiner & Altermatt, 2014; Tréguier

et al., 2014; Carim *et al*., 2016; Ikeda *et al*., 2016). However, eDNA analysis may enable us to deepen my understanding on the spatiotemporal dynamics and life-history patterns of crustacean populations.

The goal was to locate precisely the habitat of non-migratory *P. paucidens* individuals in winter in Lake Biwa and surrounding freshwater lagoons. First, a species- specific quantitative polymerase chain reaction (PCR) assay was designed to detect DNA of *P. paucidens* in environmental samples. Second, to demonstrate the presence of non-migratory populations, this assay was used to examine the distribution of overwintering populations in the shallow waters of Lake Biwa. Last, to assess whether eDNA methods can be used to estimate the density of *P. paucidens*, the quantity of *P. paucidens* DNA in eDNA samples was compared to the population densities of this species obtained through traditional sampling methods.

3.2 Materials and Methods

3.2.1 Primers, probe design, and validation

The species-specific primers and a probe were designed to detect *P. paucidens* but not *Macrobrachium nipponense*, which is the most closely related species to *P. paucidens* inhabiting Lake Biwa. First, the mitochondrial DNA (mtDNA) sequences of the 16S rRNA region of *P. paucidens* (accession numbers: KM249043–KM249065) and *M. nipponense* (accession numbers: KU235739–KU235721) were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Primer Express (version 3.0; Applied Biosystems, Foster City, California) was used to design a set of species-specific primers and a TaqMan probe, with default settings. To verify that the designed primers would not amplify DNA from closely related species with known DNA sequences, a primer basic local alignment search tool (BLAST) search with default settings was performed. In addition, the cross reactivity with 6 common freshwater shrimp species found in Japan: *Caridina multidentata*, *Neocaridina denticulata denticulata*, *Paratya compressa*, *Paratya improvisa*, *Macrobrachium*

nipponense, and *Procambarus clarkia* was checked. Total DNA was extracted from tissues with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Thereafter, quantitative PCR (qPCR) with the DNA of the 6 species and *P. paucidens* was performed as the template. Amplified products were directly sequenced using a commercial sequence service (Fasmac, Atsugi, Japan).

3.2.2 Study area

Lake Biwa is a freshwater lake in the central part of Honshu Island, Japan (lat 35°01′ N, long 136°00′ E; altitude 85 m asl). The lake is divided into north and south basins. The north basin, which corresponds to the major part of the lake, has a maximum depth of 104 m and a water volume of 27.3 km^3 (Okamoto, 1984). The south basin is small and shallow, with an average depth of 3.5 m and a water volume of 0.2 km^3 (Okuda & Kumagai 1995).

3.2.3 Detection of the distribution of non-migratory individuals

To examine the seasonal distribution of *P. paucidens* in Lake Biwa, I collected eDNA samples from 21 shore sites (E1–E21), of which 5 (E1–E3, E20, and E21) were in the south and 16 in the north basin (Figure 3.1, Table 3.1). Environmental DNA samples were also collected from 32 freshwater lagoon sites (N1–32), of which 7 (N1–N6, and N32) were close to the south basin and 25 to the north basin (Figure 3.2, Table 3.2). Coastal sampling was conducted on 9 November 2015 and 16 February 2016, and freshwater lagoon sampling was conducted on 16 November 2015 and 5 February 2016. Water from the freshwater lagoon sites tended to clog filters for volumes >500 mL, so this volume was set as the threshold for water samples. The plastic beaker was used to measure the volume of water samples, which also was used to collect samples and to pour them into disposable plastic bags. Each water sample was collected carefully from the surface water to avoid sediment resuspension that potentially influ Figureences the results of aqueous eDNA assays.

According to the on-site water filtration system described by Yamanaka *et al*. (2016b) the water samples were immediately filtered. The water samples were filtered using 47-mm glass-fiber filters (GF/F; GE Healthcare Japan, Tokyo, Japan; nominal pore size = 0.7 μm; cf. Minamoto *et al*., 2016 for selection of filter type for eDNA sampling), filters were folded inward with sterile forceps, wrapped in Al foil, and sealed in small plastic bags. Samples were kept in a cooler box filled with ice packs that had been precooled in ultralow freezers. The temperature in the cooler box was maintained at ~ – 20°C. After transportation to the laboratory, the samples were stored in a – 20°C freezer. A negative control was sampled in the field every 10 sites. Negative control samples were brought containing 500 mL of ultrapure water from the laboratory to the field site to check for unintended cross-contamination during sample transportation and filtration and during the following DNA analysis. All equipment used in water collection and DNA extraction was either disposable or decontaminated with bleach solution (diluted household bleach product containing ~0.1% sodium

hypochlorite) for >5 min before use to remove residual DNA. The decontaminated equipment was rinsed with ultrapure water before use. Disposable gloves were used in all procedures to minimize the risk of contamination.

The eDNA was extracted from filter samples with procedures published by Miya *et al*. (2015), with slight modifications. Each filter sample was removed from the Al foil, rolled into a cylindrical shape with sterile forceps, and placed in a spin column (EZ-10 Spin Column and Collection Tube; Bio Basic Inc., Ontario, Canada), from which the silica membrane had been removed. Filter samples were spun dry by centrifugation at 6000g for 1 min and incubated in a DNA extraction buffer (200 µL ultrapure water, 100 µL Buffer AL, and 20 µL proteinase K) at 56°C for 30 min. The Buffer AL and proteinase K were available as part of a DNA extraction kit (DNeasy Blood and Tissue Kit; Qiagen, Hilden, Germany). Then the column was centrifuged at 6000g for 1 min, after which the filtrate was recovered and temporarily stored in a microtube (1^{st} filtrate). TE buffer (pH 8.0, 200 µL) was added to the filter, incubated at room temperature for 1 min, and

centrifuged at 6000g for 1 min to recover the DNA still remaining on the filter (2^{nd} filtrate). The 1^{st} and 2^{nd} filtrates were mixed in a 2 mL collection tube, to which I added 200 µL of Buffer AL and 600 µL of ethanol. The mixture (final volume = ~1210 µL) was processed in a DNeasy spin column according to the manufacturer's instructions. At the final stage, DNA samples collected in 100 µL of Buffer AE were stored at – 20°C until qPCR analysis.

3.2.4 Quantification of *P. paucidens* eDNA by qPCR

The copy number of *P. paucidens* 16S rRNA was quantified by qPCR on a StepOnePlus Real-Time PCR System (Life Technologies, Foster City, California) based on 3 replicates/sample and with 3 replicates of quantification standards and negative controls. To construct a standard curve, 3.0×10^4, 3.0×10^3, 3.0×10^2 and 3.0×10^1 copies of target DNA was used as quantification standards. Each TaqMan reaction contained 900 nM each primer, 125 nM TaqMan probe, 1 Gene Expression Master Mix (Life Technologies), and 2 µL template DNA, in a total

volume of 20 μL. The qPCR conditions were: 2 min at 50 °C followed by 10 min at 95 °C and by 55 cycles of 15 s at 95 °C and 1 min at 60 °C. The average of the 3 replicates was regarded as the value of DNA concentration. When a negative detection was obtained for any of the replicates, the DNA concentration of that replicate was assigned to 0 (Ellison *et al.*, 2006). 3.0×10^4, 3.0×10^3, 3.0×10^2, 3.0×10^1, 10.0, 3.0, and 1.0 copies of target DNA were used as templates and amplified with 3 replicates. The limit of quantification was 3 copies (see Results), so a sample with calculated concentration <3 copies/reaction was treated as 'positive but under quantification limit (UQL).'

3.2.5 Validation of qPCR as a quantitative method

To test whether my qPCR method was a quantitative measure of *P. paucidens* abundance, individuals were collected with seine traps (mesh: 4 × 4 mm) at 5 freshwater lagoons, Hirako-Yanagihirako, Jinjonuma, Hasuike, Kohoku-Nodanuma, and Hamabunnuma, concurrently with eDNA sampling between 6–9

December 2016. The seine traps were installed at the in- and outflow of the 5 freshwater lagoons in the afternoon and removed them the next morning to collect *P. paucidens*. The seine traps were installed at each site during the same time (i.e., within 1⁄2 d), so the number of individuals collected by each trap was regarded as catch per unit effort (CPUE), which was used as an index of the relative abundance of *P. paucidens*.

Before installing the seine traps, 1-L water samples were collected at the in- and outflow of the 5 freshwater lagoons. Each water sample was carefully collected from surface water to avoid sediment resuspension. To prevent DNA degradation, 1 mL of 10% benzalkonium chloride solution was immediately added after collecting the water samples. The water samples were transported at ambient temperature and filtered them in the laboratory. The negative controls containing 1 L of ultrapure water were opened in the field, added 1 mL 10% (mass/volume) benzalkonium chloride solution, and brought them back to the laboratory. These

negative controls were subjected to the same experimental procedures as water samples. Filters were stored at – 20 °C until DNA extraction.

Different water sampling methods were used by the 2 universities performing the surveys. Sample volume and DNA filtration and extraction protocols slightly differed between these methods, but such differences did not influence the results (see Discussion). In this experiment, DNA extraction from filter samples was carried out using the method described by Uchii *et al*. (2016). Two filters were placed in a single Salivette tube (Sarstedt, Nümbrecht, Germany), and the DNeasy Blood and Tissue Kit were used. The tubes were incubated at 56°C for 30 min after adding 400 µL of Buffer AL and 40 µL of proteinase K. The solutions were recovered by centrifugation, added 220 µL of TE buffer to the filters, and the centrifugation procedure was repeated. 500 µL of ethanol was added to the recovered solution and Uchii *et al*. (2016) was followed in the subsequent procedures. At the final stage, DNA samples were collected in 100 µL of Buffer AE and stored at – 25 °C until qPCR. Quantification of *P. paucidens* eDNA by

qPCR was carried out under the conditions described above. During water collection and DNA extraction, precautions were taken to avoid contamination as described above.

The PCR inhibition was tested in the eDNA samples according to Jane *et al*. (2015), with slight modifications. After adding 3.0×10^4 copies of standard DNA to the eDNA samples used in the qPCR, another qPCR was performed as described above. The threshold cycle (Ct: the number of cycles required for enough amplified PCR product to accumulate that it crosses a threshold recognized by the qPCR instrumentation) of the 3.0×10^4 copy standards was compared with that of eDNA samples 1 3.0×10^4 copies of standard DNA to identify Ct shifts.

The relationship was examined between eDNA concentration and CPUE, freshwater lagoons, and sampling positions with a generalized linear model (GLM). In the model, CPUE, sites (lagoons), and sampling positions (inlet or outlet) were set as explanatory variables, and eDNA copy numbers in each PCR

as the response variable. The GLM analysis was run in R (version 3.3.2; R Project for Statistical Computing, Vienna, Austria).

3.3 Results

Primers and a probe specific for *P. paucidens* were designed (Table 3.1). I confirmed that my assay successfully amplified the 16S rRNA sequence of *P. paucidens*. The primer- BLAST search revealed that no other freshwater shrimps in Japan would be amplified with the designed primers. In addition, the qPCR showed no cross-reactivity with 6 common freshwater shrimp species in Japan. Thus, the specificity of the primers was confirmed. Amplicon sequences were confirmed as the target sequences with BLAST on the National Center for Biotechnology Information (NCBI) web service (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

In all the runs, R^2 values of calibration curves were >0.985. The ranges of slopes, y-intercepts, and PCR efficiency were between 23.639 and 23.324, 44.535

and 49.891, and 89.3 and 97.4%, respectively. The copy number of gene fragments of *P. paucidens* in each PCR (copies/reaction), and eDNA concentration in the sample water (copies/L) based on the calibration curve of each run and the Ct value of each sample were calculated. The limit of quantification was determined as 3 copies, because this was the amount of template DNA copies which could be successfully quantified with 2 of 3 replicates, and no amplification was achieved with a single copy of DNA template.

In November 2015, eDNA of *P. paucidens* was detected in 4 of the 21 shore sites. At the sites with positive detections, DNA concentrations ranged from UQL to 2.2×10^3 copies/L. In February 2016, 4 of the 21 shore sites were positive for *P. paucidens* eDNA with concentrations ranging from UQL to 1.2×10^3 copies/L (Figure 3.1). However, the eDNA positive sites were not consistent between these 2 sampling periods. In freshwater lagoons, 12 of the 32 sites were positive for *P. paucidens* eDNA in November 2015 with concentrations varying UQL to 2.2 ×

10^3 copies/L, whereas in February 2016, 9 of the 32 sites were positive for *P. paucidens* eDNA with concentrations varying UQL to 1.2×10^5 copies/L (Figure 3.2). All eDNA samples collected from shore and freshwater lagoon sites in the south basin were negative in 2015 and 2016 (Figures 3.1, 3.2). All negative controls confirmed that no contamination occurred during DNA extraction and PCR processes.

The CPUE of *P. paucidens* in the 5 freshwater lagoons varied from 3 to 87 in December 2016 (Fig. 3). All *P. paucidens* eDNA samples collected concurrently from these 5 lagoons were positive, except for the lagoon in the south basin (Figure 3.3). The eDNA concentration at positive sites varied from 1.8×10^2 to 2.1×10^3 copies/L and showed great variation within a freshwater lagoon. Sampling position (inlet or outlet) and CPUE significantly affected eDNA concentration (GLM; $p < 0.01$ for both variables), but sampling sites (lagoons) did not (GLM; $p > 0.05$).

Results of the PCR inhibition test showed no Ct shift ($\Delta Ct < 1$), suggesting that no PCR inhibition biased the results.

3.4 Discussion

Previous investigators reported that most *P. paucidens* populations in Lake Biwa migrate to deep waters in autumn where they overwinter at high density (Harada 1966; Nishino 1983). My eDNA approach revealed the existence of non-migratory individuals that overwinter in shallow shores and freshwater lagoons of Lake Biwa. Some fish show different life-history traits within the same species (Haryu, 1992; Ito *et al*., 2015). The Ayu-fish (*Plecoglossus altivelis*) from Lake Biwa, for example, can be classified into 2 groups based on their migration timing: one group migrates before late spring and the other migrates after late spring (Azuma, 1970, 1973). However, knowledge of non-migratory individuals of *P. paucidens* in Lake Biwa is limited. By precisely locating the habitat of non-migratory *P. paucidens* individuals, my study might help future research on these populations.

To date, many eDNA studies of fish and amphibians have documented a positive association between eDNA quantity and abundance of an organism. However, few researchers have investigated this aspect in crustaceans (Tréguier *et al*., 2014; Carim *et al*., 2016). I surveyed a large open-water area of Lake Biwa and succeeded in detecting small *P. paucidens* individuals. Many endemic invertebrates inhabit this lake, and the further development of eDNA technology can promote understanding of their ecology and life history.

Palaemon paucidens eDNA was extensively detected in shallow shore waters and freshwater lagoons of the north basin in winter, but not in the 2 surveys conducted in the south basin. The population density of *P. paucidens* probably is very low in this basin during the winter season, as shown in the Hirako-Yanagihirako lagoon, where only a few individuals were collected by the seine traps. One possible reason for this very low abundance is predation by invasive carnivorous fishes, such as the Largemouth Bass *Micropterus salmoides* and the Bluegill *Lepomis macrochirus*, which have propagated exponentially in the south

basin (Kuwamura, 2001; Nakajima *et al.*, 2001). A combination of dietary analysis and qPCR of fecal DNA revealed that Large- mouth Bass have a strong feeding preference for *P. paucidens* (Sugiura & Taguchi, 2012). Terashima (1980) also reported that Bluegill preferred to feed on shrimps after colonizing this lake.

The PCR inhibition were tested with the 5 lagoon samples used for qPCR and found that no PCR inhibition biased the results and that negative results obtained for 3 eDNA samples were not caused by PCR inhibition. Turner *et al.* (2015) reported that eDNA is heterogeneously distributed in the environment or occurs in clumps. Thus, eDNA concentration obtained might be very low in the water samples. In future studies, detection probabilities or quantified copies of eDNA may increase if larger volumes of DNA are tested.

Environmental DNA detection via qPCR as a quantitative measure of the relative abundance of *P. paucidens* was validated by my study. In many eDNA studies on fish and amphibians, the eDNA concentration reflects the relative abundance of their local populations (Takahara *et al.*, 2012; Pilliod *et al.*, 2014;

Yamamoto *et al.*, 2016). However, the spatial scale at which such a quantitative approach based on eDNA can be effective for estimating species' or populations' relative abundance is still controversial. In stream ecosystems, Jane *et al.* (2015) reported that eDNA is homogenized in a 30 m downstream movement of the water body from its upstream source under a high-flow regime. In closed natural water bodies, Dunker *et al.* (2016) found that DNA of Northern Pike slowly diffused away from the source, such that the probability of detecting DNA decreased with distance. This result suggests that water sampling in rivers during high-flow or high-wind events would increase the chance of collecting eDNA resuspended from sediment beds (Evans, 1994; Jamieson *et al.*, 2005). In my study, each freshwater lagoon is >100 m, and the lake current is very weak (Biological Resource Research Team in Lake Biwa, 1966; Nishino, 2008). Moreover, to avoid sediment resuspension that may influence the results of aqueous eDNA tests, the water samples were carefully collected. Therefore, through my considering, the detections were not from eDNA attached to sediment particles, homogenization of

P. paucidens eDNA within the freshwater lagoon is unlikely, and each eDNA sample may be considered independent. In fact, eDNA concentrations in each freshwater lagoon showed great spatial variation that was accompanied by large variations in *P. paucidens* CPUE values, suggesting that the method used here can be a quantitative measure of *P. paucidens* abundance at the local scale. The quantitative method may be applicable to other small crustacean species, increasing the potential for crustacean research and increasing my understanding of their ecology and life history in nature.

Two sampling methods were used in which the volume of samples and the methods of DNA filtration and DNA extraction were slightly different. Yamanaka *et al*. (2017) reported that eDNA yields between these 2 methods show nonsignificant differences and, therefore, the differences between these 2 methods should not influence the results. However, the limits of quantification were different between sample series because of varying sample volumes. Therefore, the results between the 2 sampling series were not compared.

Overall, the existence of non-migratory *P. paucidens* winter populations in Lake Biwa was successfully confirmed because eDNA was collected at shore and freshwater lagoon sites during winter. However, in future studies, eDNA surveys should be conducted in other seasons and sites, including offshore and lake-bottom samples, to comprehensively understand the life history polymorphism of *P. paucidens* populations in Lake Biwa.

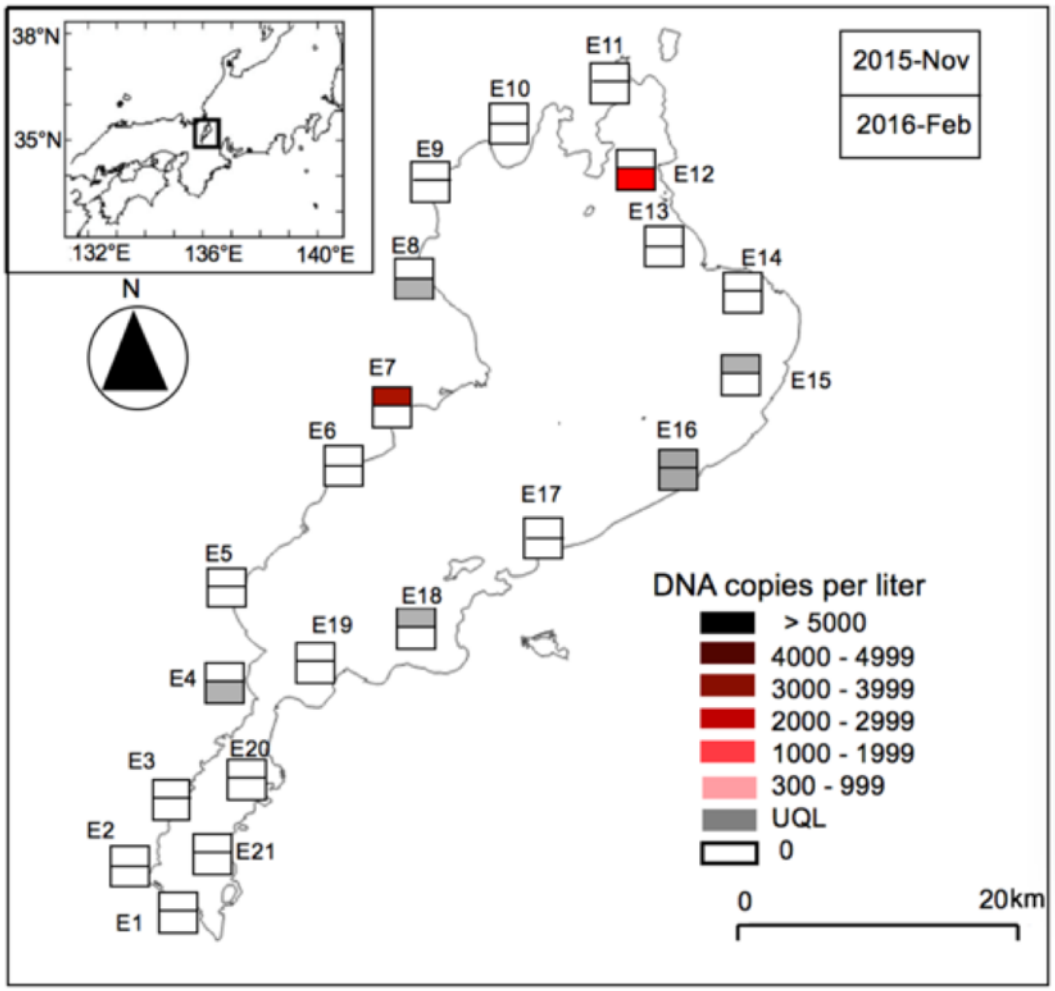

Figure 3.1. Locations of sampling sites along the shore of Lake Biwa and environmental DNA (eDNA) quantification for each sampling site. Boxes show the eDNA concentrations of *Palaemon paucidens* in November 2015 (upper part) and February 2016 (lower part). UQL = under quantification limit.

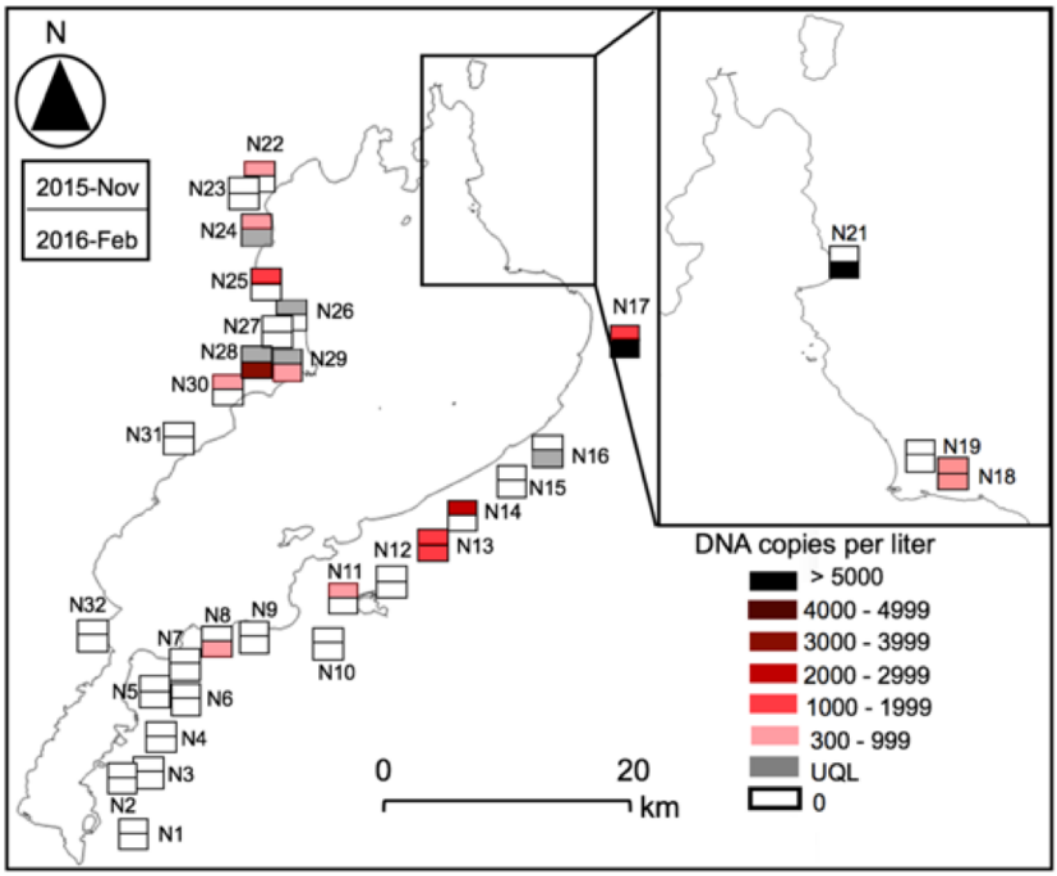

Figure 3.2. Location of the sampling sites within the freshwater lagoons of Lake Biwa and environmental DNA (eDNA) quantification for each sampling site. Boxes show the eDNA concentrations of *Palaemon paucidens* in November 2015 (upper part) and February 2016 (lower part). UQL = under quantification limit.

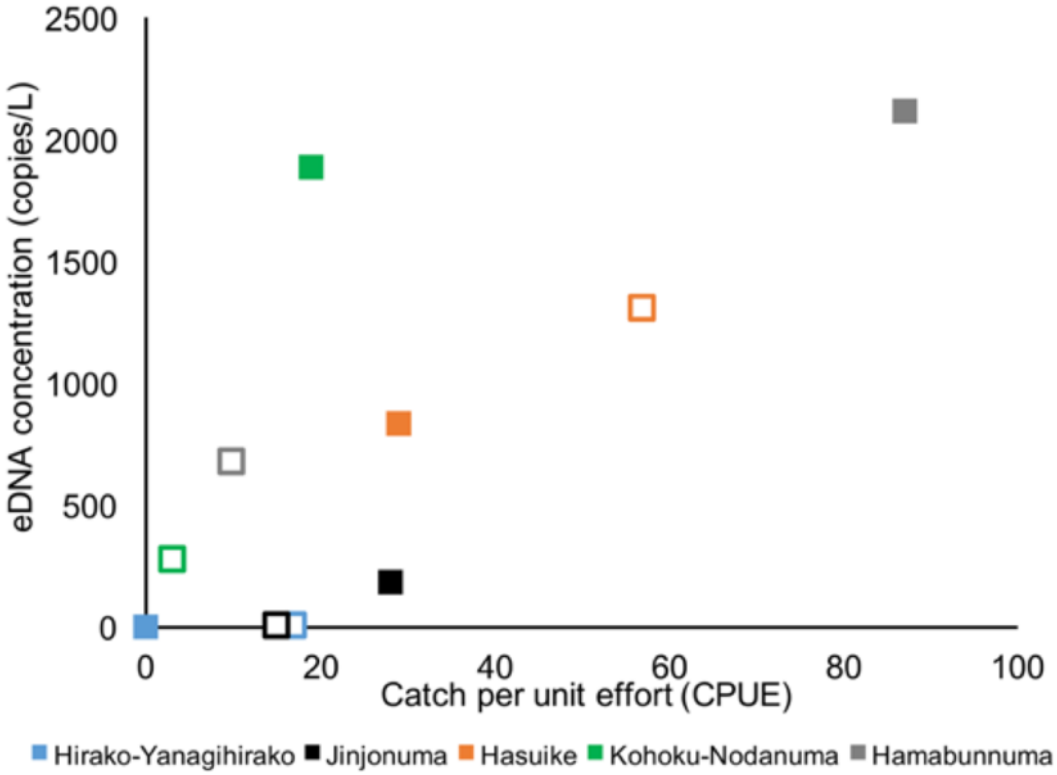

Figure 3.3. Relationships between the concentration of environmental DNA (eDNA) and catch per unit effort (CPUE; number of *Palaemon paucidens* captured/. d by seine net collection) in Lake Biwa freshwater lagoons. The closed and open boxes denote out- and inflow sites, respectively. The eDNA concentration was positively correlated with CPUE values (GLM, $p < 0.01$).

Table 3.1. Primers, probe and plasmid used in my study.

Name	**Sequence (5' -3')**
Palaemon paucidens forward primer	AAGTCTAACCTGCCCACTGAGTTA
Palaemon paucidens reverse primer	TTTAAGCCTTTTCACTTAAAGGTCA
Palaemon paucidens probe	FAM-ATGAGGGAAAAACTG-NFQ-MGB

Table 3.2. Location details and quantitative real-time PCR (qPCR) results obtained for the water samples collected in Lake Biwa shore sites. The qPCR results show the number of positives in the 3 replicates. Environmental DNA (eDNA) concentration was assessed by qPCR. + indicates eDNA of *P. paucidens* was detected but was below quantification limit.

				qPCR results		eDNA concentration (copies/L)	
Site	Location	Latitude	Longitude	Nov 2015	Feb 2016	Nov 2015	Feb 2016
E1	Gotenhama, Otsu City	34°59'20"	135°53'54"	0	0	0	0
E2	Yanagasaki, Otsu City	35°1'50"	135°52'2	0	0	0	0
E3	Hieitsuji, Otsu City	35°4'9"	135°53'16"	0	0	0	0
E4	Mano, Otsu City	35°7'37"	135°55'17"	0	1	0	+
E5	Hachiyado, Otsu City	35°11'15"	135°55'3"	0	0	0	0
E6	Kitakomatsu, Otsu City	35°15'13"	135°58'27"	0	0	0	0
E7	Nagata, Takashima City	35°98'9"	136°1'35"	3	0	2239	0
E8	Shinasahi-cho, Takashima City	35°22'32"	136°2'44"	0	1	0	+
E9	Makino-cho, Takashima City	35°26'28"	136°2'44"	0	0	0	0
E10	Nishiazaicho, Nagahama City	35°28'38	136°6'43"	0	0	0	0
E11	Kinomoto-cho, Nagahama City	35°28'44"	136°6'47"	0	0	0	0
E12	Kohoku-cho, Nagahama City	35°27'0"	136°11'21"	0	3	0	1200

E13	Ohama-cho, Nagahama City	35°24'11"	136°12'54"	0	0	0	0
E14	Tamura-cho, Nagahama City	35°21'26"	136°16'40"	0	0	0	0
E15	Iso, Maibara City	35°18'14"	136°15'24"	2	0	+	0
E16	Hassaka-cho, Hikone City	35°15'29"	136°12'36"	1	2	+	+
E17	Shingai-cho, Hikone City	35°13'30"	136°8'59"	0	0	0	0
E18	Chomeiji-cho, Omihachiman City	35°9'32"	136°3'36"	1	0	+	0
E19	Yoshikawa, Yasu City	35°8'28"	135°59'1"	0	0	0	0
E20	Oroshimo-cho, Kusatsu City	35°4'10"	135°56'4"	0	0	0	0
E21	Kitayamada-cho, Kusatsu City	35°1'56"	135°54'46"	0	0	0	0

Table 3.3. Location details and quantitative real-time PCR (qPCR) results obtained for the water samples collected in Lake Biwa freshwater lagoons. The qPCR results show the number of positives in the 3 replicates. Environmental DNA (eDNA) concentration was assessed by qPCR. + indicates eDNA of *P. paucidens* was detected but below quantification limit.

				qPCR results		eDNA concentration (copies/L)	
Site	Location	Latitude	Longitude	Nov 2015	Feb 2016	Nov 2015	Feb 2016
N1	Tonotagawa-naiko	34°59'43"	135°54'36"	0	0	0	0
N2	Yanagidairako	35°02'58"	135°55'34"	0	0	0	0
N3	Hirako	35°03'00"	135°55'12"	0	0	0	0
N4	Shinanaka-naiko	35°03'31"	135°56'51"	0	0	0	0
N5	Erinohama	35°05'02"	135°56'52"	0	0	0	0
N6	Kinohama-naiko	35°05'33"	135°56'23"	0	0	0	0
N7	Yasu-gawa River	35°07'08"	135°57'50"	0	0	0	0
N8	Yasu-gawa River	35°07'29"	135°59'30"	0	1	0	466
N9	Yasu-gawa River	35°08'33"	135°03'13"	0	0	0	0
N10	Kitazawanuma	35°08'28"	136°00'20"	0	0	0	0
N11	Kitanoshosawa	35°08'54"	136°05'34"	1	0	476	0
N12	Nishinoko	35°09'48"	136°06'20"	0	0	0	0

N13	Iba-naiko	35°11'17"	136°08'11"	1	2	1180	1363
N14	Jinjonuma	35°13'44"	136°09'28"	3	0	2212	0
N15	Sonenuma	35°14'36"	136°11'35"	0	0	0	0
N16	Hikone-Nodanuma	35°14'58"	136°12'36"	0	1	0	+
N17	Hasuike	35°19'09"	136°16'09"	3	3	1479	5920
N18	Hosoe-naiko	35°23'16"	136°14'36"	1	1	482	306
N19	Minamiura-naiko	35°24'41"	136°12'33"	0	0	0	0
N20	Hayasaki-naiko	35°25'03"	136°12'14"	0	0	0	0
N21	Kohoku-Nodanuma	35°27'05"	136°11'48"	0	3	0	172248
N22	Nukigawa-naiko(north)	35°25'56"	136°02'21"	1	0	892	0
N23	Nukigawa-naiko(south)	35°25'46"	136°02'25"	0	0	0	0
N24	Hamabunnuma	35°25'22"	136°02'42"	1	1	544	+
N25	Harieokawa	35°21'54"	136°03'16"	2	0	1025	0
N26	Suganuma	35°20'56"	136°04'07"	1	0	+	0
N27	Ekainuma	35°19'16"	136°03'32"	0	0	0	0
N28	Gotandanuma	35°19'09"	136°02'45"	2	3	+	2467
N29	Matsunoki-naiko	35°18'47"	136°03'14"	2	2	+	583
N30	Otomegaike	35°17'31"	136°00'52"	3	0	834	0
N31	Oumimaikonuma	35°14'23"	135°57'57"	0	0	0	0
N32	Katata-naiko	35°06'53"	135°55'21"	0	0	0	0

Chapter 4. Polymorphic migratory behavior in a panmictic population of *Palaemon paucidens* in Lake Biwa

4. 1 Introduction

Life history polymorphism reveals that individual members of a group exhibit different life history traits (Gross, 1996; Sakei *et al*., 1999). This has been observed in certain species of reptiles (Hatase *et al*., 2004), birds (Berthold, 1991; Rolshausen *et al*., 2009), fish (Bernatchez & Dodson, 1990; Foote *et al*., 1989) and insects (Danks, 1994). Behavioral ecologists have confirmed the genetic basis of behavioral polymorphism (Fitzpatrick *et al.*, 2005; Bell & Robinson, 2011; Zayed & Robinson, 2012). However, in some cases, there are many factors for behavioral polymorphism, such as the environmental changes of habitats and the existence of natural enemies (Koizumi *et al*., 2006). Thus, evaluating the contribution of genetic and non-genetic factors for behavioral polymorphism is essential to understand the ecological and evolutionary origin of life history polymorphism.

This study focused on the freshwater shrimp *Palaemon paucience* (Crustacea, Decapoda, Palaemonidae) in Lake Biwa (35°01′ N, 136°00′ E, Figure

4.1), which exhibit a polymorphism in seasonal migration behavior (Chapter 3, 5). Lake Biwa is the largest freshwater lake in Japan, and the third oldest lake in the world (Nishino & Watanabe, 2000). Lake Biwa includes streams and freshwater lagoon connected to the main lake, which play an important role for the aquatic organisms inhabiting therein. Some aquatic organisms move between the main lake, freshwater lagoon, paddy fields and stream (Nakajima *et al.*, 2001). *Palaemon paucience* exhibits seasonal migration within the lake: it is abundant in shallow waters from spring to summer, then most individuals (called migratory individuals in this study) move to offshore deep waters (about 90 m in depth) for autumn and winter, and they migrate again from the offshore deep areas to shallow waters in spring (Harada 1966; Nishino 1983). Meanwhile, the results in Chapter 2 showed that some individuals (referred to as non-migratory individuals in this study) spend winter in the shallow shores and freshwater lagoons of Lake Biwa. However, the

factors relating to the observed polymorphism in migratory behavior are currently unknown for this species.

Polymorphic migratory behavior has been intensively studied in *Oncorhynchus masou* (Gross, 1996; Sakei *et al*., 1999; Koizumi *et al.*, 2006). It is pointed out that small-sized and well-nourished male individuals are successful in the competition with peers and stay in the river for a lifetime, while small male individuals in poor nutritional status which failed in the competition move to the sea, grow up therein, and return to the river for reproduction. These polymorphic migratory behaviors are directly related to subsequent reproductive success (Fleming & Gross, 1992). In *P. paucience*, the results in Chapter 2 showed that females accounted for a large proportion of the migratory individuals in Lake Biwa, but the sex ratio of non-migratory individuals is still unknown. Since migratory individuals obtain nutrients in the bottom of Lake Biwa in winter for subsequent reproduction, it is hypothesized that most of the small individuals, especially poor nutritional females, move to the bottom of the lake while some of

the well-nourished individuals stay on the shore in winter. This hypothesis predicts that the proportion of females in migratory individuals will be higher than that in non-migratory individuals in winter. In addition， previous studies have shown that *P. paucidens* feed at the bottom of the lake in winter and store nutrients for later reproduction (Chapter 2). If individuals can maintain sufficient nutrients for reproduction before winter, they would not migrate to the bottom for avoiding its costs. Thus, it is hypothesized that the nutritional condition of individuals determines migratory behavior. This hypothesis predicts that nutritional status of non-migratory individuals is better than that of migratory individuals.

To examine the genetic and non-genetic factors involved in the polymorphic migratory behavior of *P. paucience* in Lake Biwa, this study aimed to reveal genetic and ecological differences between two migratory phenotypes. First, a population genetic analysis was performed using microsatellite markers to elucidate the genetic structure of the *P. paucidens* populations in Lake Biwa.

Then phenotypic characteristics, including sex ratio, body size and fatness related to nutritional condition, were analyzed. In addition, carbon and nitrogen stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) were analyzed for the purpose of understanding the food source and trophic level of the polymorphic migratory phenotypes.

4.2 Materials and Methods

4.2.1 Microsatellite analysis

4.2.1.1 Sample collection

To examine the genetic difference of two migratory phenotypes, samples were collected from deep (migratory) and shallow (non-migratory) waters in winter. Migratory individuals were collected from three deep water sites: S1, -40m (14 November 2014), S2, -60m (16 January 2015), and S3, -90m (24 March, 2017) (Figure 4.1; Table 4.1). Non-migratory individuals were collected from nine shallow sites: one shallow shore (S4; 24 March, 2017), the in- and outflow of four freshwater lagoons namely, Jinjonuma (S5), Hasuike (S6), Nodanuma (S7) and

Hamabunnuma (S8) on 8-9 December 2016. In addition, summer samples were also collected from nine shallow sites, which may include both non-migratory and migratory individuals: S9, close to S1 (4 August 2015), S10, close to S2 (16 September 2015) and S11 (29 July 2016), Jinjonuma (S5), Hasuike (S6), Nodanuma(S7), Hamabunnuma (S8), Yanagihirako (S12), and Katada (S13) on 28- 30 June 2016 (Figure 4.1; Table 4.1). Trawl net, basket traps and seine traps were used to catch shrimps from deep (< -40 m), shallow (> -15 m) and freshwater lagoons sites, respectively (Table 4.1). Each sample was stored at –20°C for further analyses. A total of 520 individuals from 22 sites were collected throughout the study period.

4.2.1.2 Microsatellite genotyping

A total of 520 individuals from 22 sites were used for population genetic analysis. Total DNA was extracted from muscular tissues using Wizard® Genomic DNA Purification Kit (Promega) and stored in 50 µL of TE buffer at 4 ˚C. Six

microsatellite loci (PAU0133, PAU0316, PAU0818, PAU0930, PAU0329, PAU0431) developed by Song *et al*. (2009) were used for microsatellite genotyping. Polymerase chain reaction amplifications were conducted in a final reaction volume of 10 µL using AmpliTaq Gold® 360 Master Mix (Life Technologies), a final concentration of 10 nM forward and reverse primers. The PCR cycling started with an initial denaturation at 95 °C for 10 min; 32 cycles of denaturation at 95 °C for 30 s, annealing at a temperature depending on the locus for 90 s, extension at 72°C for 90 s; and a final extension at 60 °C for 30 min. PCR products were analyzed by ABI3130 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA) using the internal size standard (GeneScan™ 400HD ROX™ dye Size Standard; Foster City, CA, USA). The size of the amplified fragments was determined using GeneMapper ver. 3.0 software (Applied Biosystems).

4.2.1.3 Population genetic analysis

Observed (H_o) and expected (H_e) heterozygosities were calculated for each locus within each sample, and the deviation from Hardy-Weinberg equilibrium (HWE) was examined by a randomization test with 999 pseudoreplications. The genetic differentiation between samples were evaluated by F_{ST} and D_{est} (Jost, 2008). Genetic differentiation from migratory and non-migratory individuals collected in winter were also examined by the analysis of molecular variance (AMOVA). These analyses were performed using the software GenALEx version 6.5 (Peakall & Smouse, 2012).

The genetic structure was also investigated using a Bayesian model-based clustering method implemented in the software STRUCTURE v2.3.4 (Pritchard *et al.*, 2000). All the analyses were conducted using a discarded burn-in of 10,000 steps, followed by 100,000 Markov Chain Monte Carlo (MCMC) steps. Ten independent runs were performed for each cluster K (K = 1 to K = 26). To determine the potential number of genetic groups (K), the log-likelihood profile across varied K values (K = 1 - 26) as well as Evanno's ΔK (Evanno, Regnaut &

Goudet, 2005) were computed using STRUCTURE HARVESTER v.0.6.8 (Earl & von Holdt, 2012).

4.2.2 Morphometric analysis

To compare the morphometric characteristics with migratory (from offshore in winter; S1 – S3, Figure 4.1; Table 4.1) and non-migratory individuals (from shallow sties in winter; S4 – S8, Figure 4.1; Table 4.1), a total of 269 individuals from eight sites were analyzed.

Prior to measuring morphologies, individuals were sexed with respect to the presence of an appendix masculina on the second pleopod of endopod, which is specific to the male (Bruce, 1989). Then, carapace length (CL), body length (BL) and total length (TL) were measured as a distance from the base of the eyestalk to the posterior margin, that from the eyestalk to the tip of the telson, and that from the tip of the rostrum to the tip of the telson, respectively, to the nearest 0.1 mm using a vernier scale. Dry weight of an individual (W) was measured using

a top loading electronic balance (Mettler Toledo, AG 204) to the nearest 0.1 mg. Body fatness which was calculated using Fulton's condition factor (CF, Nash *et al.*, 2000) was calculated as defined by the following equation:

$$CF = 100 \times (W / TL^3)$$

The reproductive status of females was checked by consulting the abdominal exoskeleton, which is deformed after spawning and useful in distinguishing reproduced females from others (Chapter 5). The migratory females were divided into three groups: before-reproduction female, post-spawning female, and carrying eggs female. Only before-reproduction female was found in the migratory individuals.

After measuring, a little muscle tissue was stored in 99 % ethanol and kept in a freezer (-20 °C) for genetic analysis as described above. Other muscle tissue was dried at 60 °C for 24 h for stable isotope analysis as described below.

In order to investigate the gender composition of migratory and non-migratory individuals, difference in sex ratio between migratory and non-

migratory individuals was examined using a generalized linear mixed model (GLMM) with a binomial distribution, in which the proportion of female was used as the response variable, the type of migratory behavior as an explanatory variable, and collection site as a random effect.

In addition, differences in CL between individuals with different migratory behaviors, GLMMs with a normal distribution was used. In the model, each of CL was used as response variables, and four categories of individuals, migratory male, migratory female, non-migratory male and non-migratory female were used as explanatory variables, with collection site as a random effect. Because there were no egg-bearing females and post-spawning females in the non-migratory individuals, and these individuals appeared in the migratory females at different period. In order to eliminate the differences caused by these individuals, some migratory female individuals were excluded, including egg-bearing females, and post-spawning females in this model.

To compare W and CF between sexes and between individuals with different migratory behaviors, GLMMs with a normal distribution was used. In the model, each of W or CF was used as response variables, and four categories of individuals, migratory male, migratory female, non-migratory male and non-migratory female were used as explanatory variables, with collection site as a random effect. In order to avoid the difference mentioned above, some migratory female individuals were excluded, including reproduced females and carrying eggs females in this model.

4.2.3 Stable isotope ratio

The dried muscle samples were pulverized and immersed in 2:1 chloroform-methanol solution for 24 h to remove the lipids (Bligh & Dyer, 1959). Carbon and nitrogen stable isotope ratios were performed with a Delta V Advantage mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) connected to a Flash EA 1112 elemental analyzer (Thermo Fisher Scientific) through a Conflo IV

interface (Thermo Fisher Scientific) at Ryukoku University, Japan. Isotopic notations of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) were expressed as per mil-deviation from the standards (atmospheric N_2 gas for nitrogen and PeeDee belemnite carbonate for carbon) as defined by the following equation:

$$\delta^{13}C \text{ or } \delta^{15}N = \frac{(Rsample - Rstandard)}{Rstandard} \times 1000\ (‰),$$

where R is $\delta^{15}N$ / $\delta^{14}N$ or $\delta^{13}C$ / $\delta^{12}C$. The analytical errors of $\delta^{13}C$ and $\delta^{15}N$ values were ± 0.1‰.

The carbon and nitrogen stable isotopes ratios were compared between non-migratory and migratory individuals by Wilcoxon signed rank test. One-way analysis of variance was used to compare the differences between carbon and nitrogen stable isotope ratios and sampling sites from non-migratory individuals collected by five shallow waters. The same method, is adapted to see migratory individuals collected from three deep areas. All statistical analyses were performed using R (version 3.3.2; R Project for Statistical Computing, Vienna, Austria).

4.3 Results

4.3.1 Population genetic analysis

Mean heterozygocity (Ho) within populations ranged from 0.62 to 0.76, and mean expected heterozygosity (He) within populations ranged from 0.67 to 0.82 (Table 4.1). Deviations from Hardy-Weinberg Equilibrium (HWE) were not found within any samples (Table 4.1). Pairwise F_{ST} among populations ranged from 0.008 to 0.046, and D_{est} among population ranged from -0.036 to 0.048, in which 27 and 20 of 231 estimates were significantly differed from zero, respectively (Table 4.2). Genetic differentiation among all sites was not significant (F_{ST}= 0.035; D_{est}=0.001; P >0.05). Result of AMOVA showed that only 0.02% of the total genetic variance was assigned to among the two groups, and this differentiation was not significant (P =0.46; Table 4.3).

Although Evanno's method indicated that the most likely number of populations was four (Figure 4.2), the log-likelihoods were constantly highest

from K =1 to 4. The bar plot by STRUCTRE indicated no clear genetic differentiation when assuming K = 4, suggesting that the analyzed samples consisted of a single panmictic population (Figure 4.3).

4.3.2 Morphological characteristic

The proportion of females in non-migratory individuals was significantly lower than that in migratory individuals (GLMM, p= 0.017, Figure 4.4, Table 4.4).

Migratory females, migratory males, non-migratory females and non-migratory males had CL ranging from 2.5 mm to 12.5 mm, 2.5 mm to 10.0 mm, 5.0 mm to 12.5, and 5.0 mm to 12.5 mm, respectively. Carapace length of non-migratory females was significantly greater than others (GLMM, p<0.0001, Figure 4.5A, Table 4.5a). Conditional factor was significantly greater in non-migratory females and males than in migratory females and males (Figure 4.5B, Table 4.5b). The weight of non-migratory females was significantly greater than

others, among which there was no difference in their weight (Figure 4.5C, Table 4.5c).

4.3.3 Stable isotope ratio

The nitrogen stable isotope ratio of the non-migratory individuals was significantly lower than that of migratory individuals ($P < 0.0001$; Figure 4.6A). Carbon isotopes was also significantly lower than that of migratory individuals ($P = 0.0007$; Figure 4.6B).

Stable isotope ratios of non-migratory individuals significantly differed among sites ($\delta^{15}N$, $F_{4, 69}=17.87$, $P<0.0001$; $\delta^{14}C$, $F_{4, 69}=8.54$, $P<0.0001$; Figure 4.7), while those of migratory individuals did not differ among sites ($\delta^{15}N$, $F_{2, 17}=0.07$, $P=0.93$; $\delta^{13}C$, $F_{2, 17}=3.12$, $P=0.073$; Figure 4.7).

4.4 Discussion

The results of population genetic analyses indicated that *P. paucidens* in Lake Biwa consists of a large panmictic population. Thus, migratory and non-migratory individuals reveal a behavioral polymorphism within a population, and are not the members of genetically differentiated populations. This implies two possibilities of genetic basis of the polymorphic migratory behavior in *P. paucidens*. First, the polymorphism in migratory behavior may have a simple genetic basis (i.e., determined by a single locus or a tightly-linked genomic region). If this is true, it is expected that balancing selection play a role in maintaing allele polymorphism responsible for migratory behavior. Second, the polymorphism in migratory behavior may have no genetic basis, and depends only on ecological and/or environmental factors. Previous studies have pointed out that the dimorphic migration behavior observed within a population is not genetic dimorphism but phenotypic plasticity in accordance with the growing condition (Jonsson & Jonsson, 1933; Tamate & Yamamoto, 2004).

Two migratory phenotypes were indicated to differ in sex ratio, body size and body condition. The proportion of females in migratory individuals was higher than in non-migratory females, and only migratory female individuals involved post-spawning females (Figure 4.4). Non-migratory females was significantly larger than others (Figure 4.5A). CF were significantly lower in migratory individuals than in non-migratory individuals (Figure 4.5B). These results suggested that migratory individuals, especially post-spawning females, showed poor growth or nutritional condition compared with non-migratory individuals. These results support the conditional strategy hypothesis.

Carbon and nitrogen isotopes ratios of migratory individuals were significantly higher than non-migratory individuals (Figure 4.6 A, B). Interestingly, there was no significant difference in $\delta^{13}C$ values across the sampling sites at the bottom of the lake in winter, while there were significant differences in $\delta^{13}C$ values between sampling sites in shallow shores in winter (Figure 4.7). These results suggested that a same food source was shared in deep

sites, but there were different food sources at different sampling sites in shallow sites in winter.

Similar to the case of *O. masou*, *P. paucidens* showed that small individuals with poor nutritional condition migrated. Unlike *O. masou* whose males only were differentiated into migrants or residents, *P.paucidens* in Lake Biwa involved migrants of both sexes in which only females' migration depended on growth and nutritional condition. Female individuals have larger body sizes than males in order to produce maximum fecundity (Fleming & Gross, 1992). By contrast, there may be little advantage for males to increase body size. Since large body size increases predation risk (Nakazawa *et al*., 2007; Rojas & Ojeda, 2010), optimal body size may differ between males and females. Consequently, most of the female *P. paucidens* in Lake Biwa move to the bottom of the lake for feeding and for subsequent reproduction (Chapter 2).

The two migration phenotypes of this species in Lake Biwa are possibly conditional strategies that depend on the shrimp's growth, nutrition, and

reproductive status, i.e., whether they have previously reproduced or not. However, shrimps' abdominal characteristics indicating the experience of spawning may be disappear after molt. Thus, it is possible that non-migratory females are alive more than one year and the signature of spawning disappear due to growing and subsequent molt. In future studies, the age structure of the population in Lake Biwa, as well as the age of non-migratory individuals, is necessary to be determined.

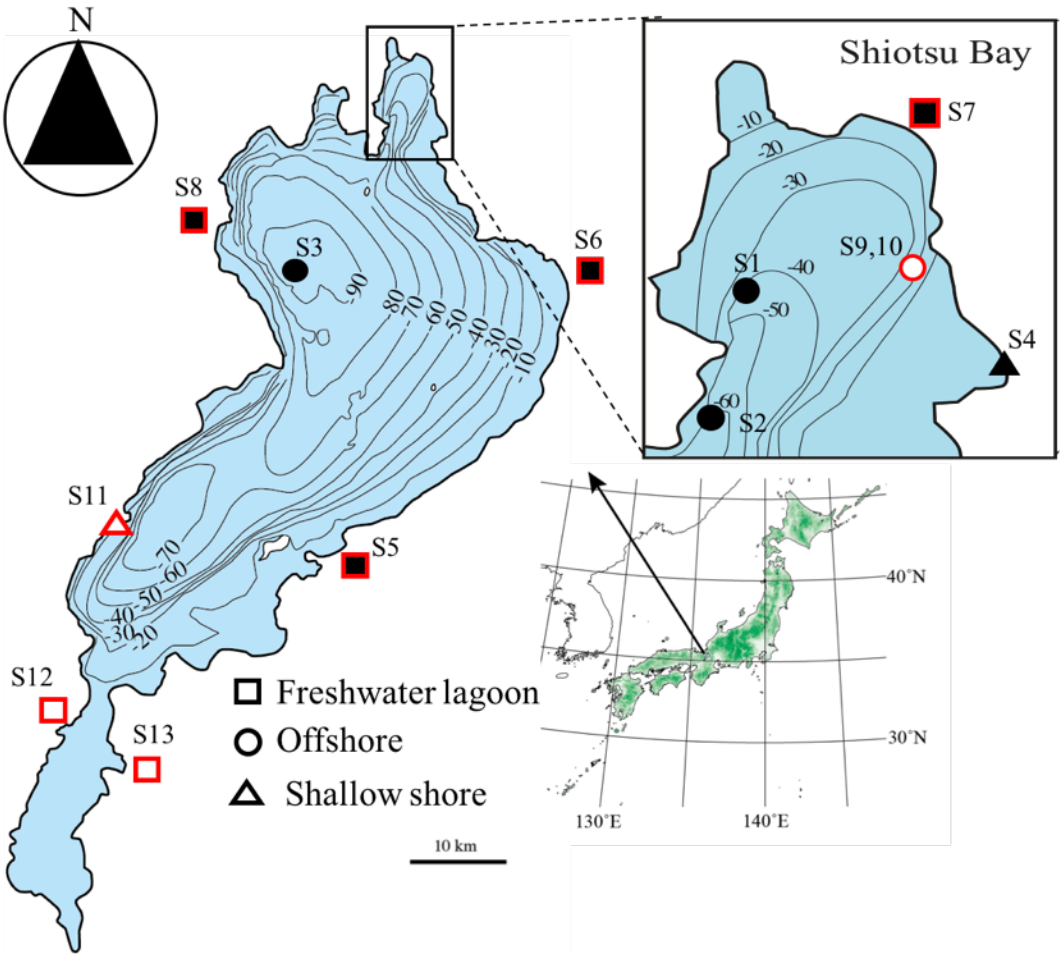

Figure 4.1. Map of the study sites. The solid legend show sample collected in winter. Circles represent migratory individuals, and squares represent non-migratory individuals. The open legend show sample collected in summer. Samples that were blacked with red circles were collected in both winter and summer.

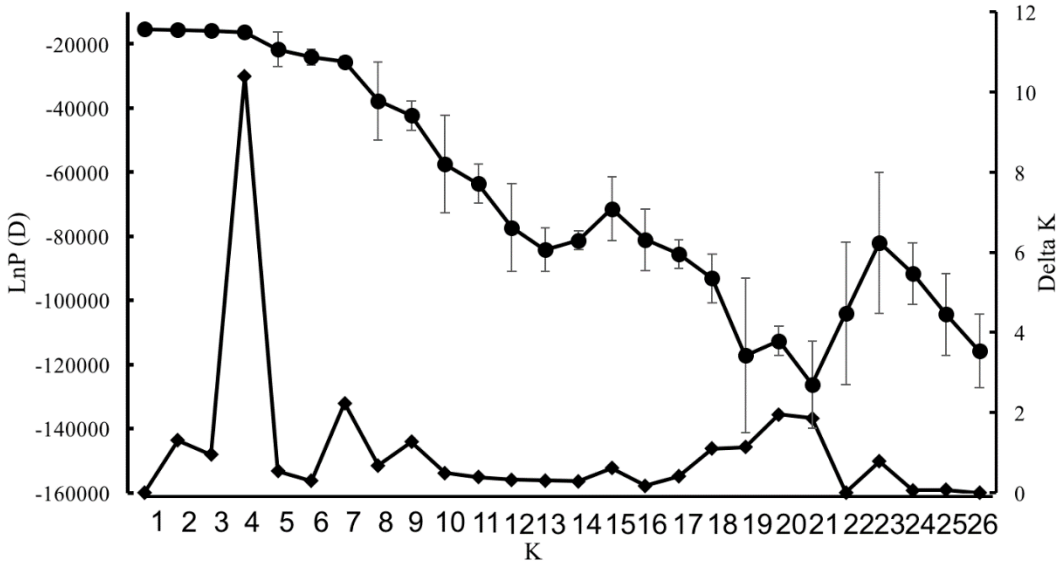

Figure 4.2. Bayesian support for a tripartite structure. Mean posterior probability Ln P (D) with standard deviance (bars) and delea K, after 10 runs for K population clusters in STRUCTURE. The analysis is for all shrimp (n= 483), without prior sample information and with an admixture model.

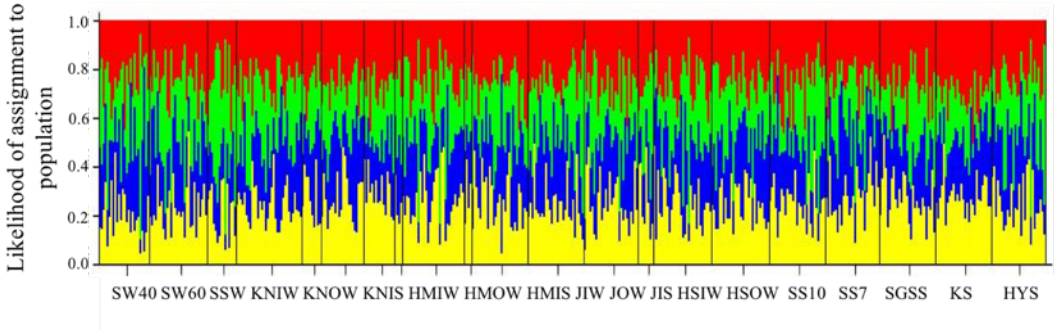

Figure 4.3. STRUCTURE bar plot for *Palaemon paucidens* collection sites in the Lake Biwa. STRUCTURE runs were completed without apriori populations assigned, admixture, and correlated alleles were assumed, and shown for the most probable number of genetic populations, K = 1. Sample location codes are as in Figure 4.2.

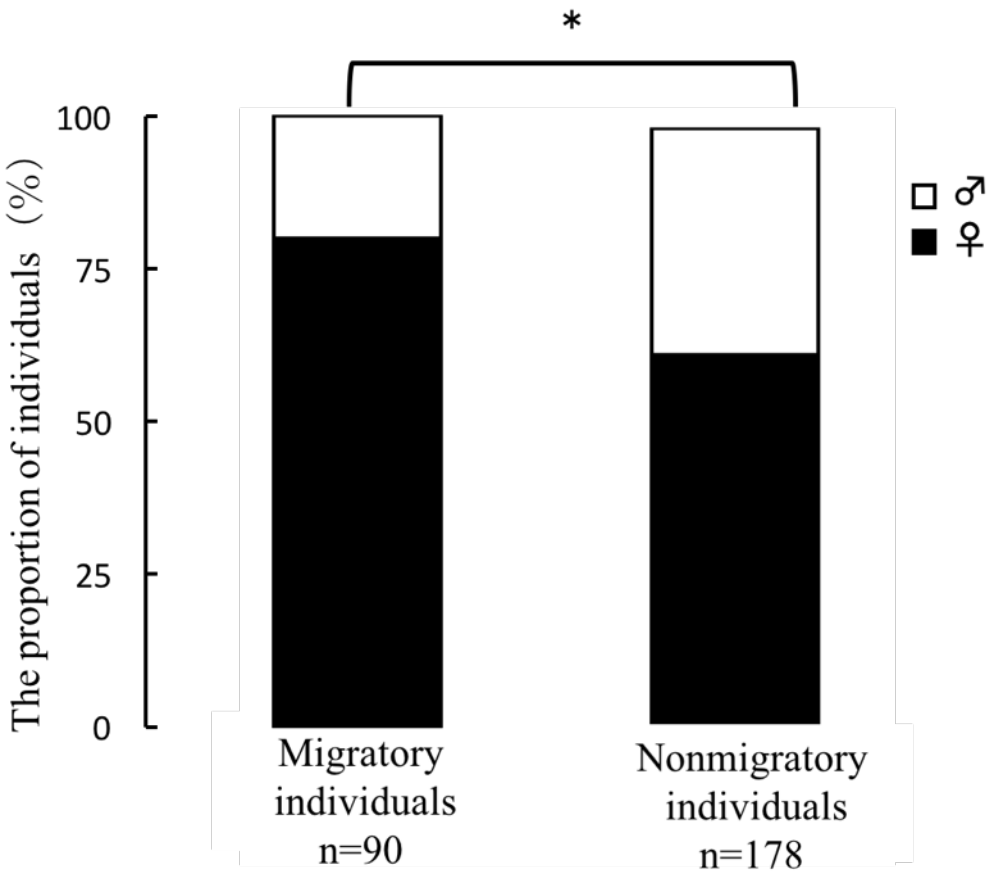

Figure 4.4. The sex ratio of non-migratory and migratory individuals. Black show female, white show male.

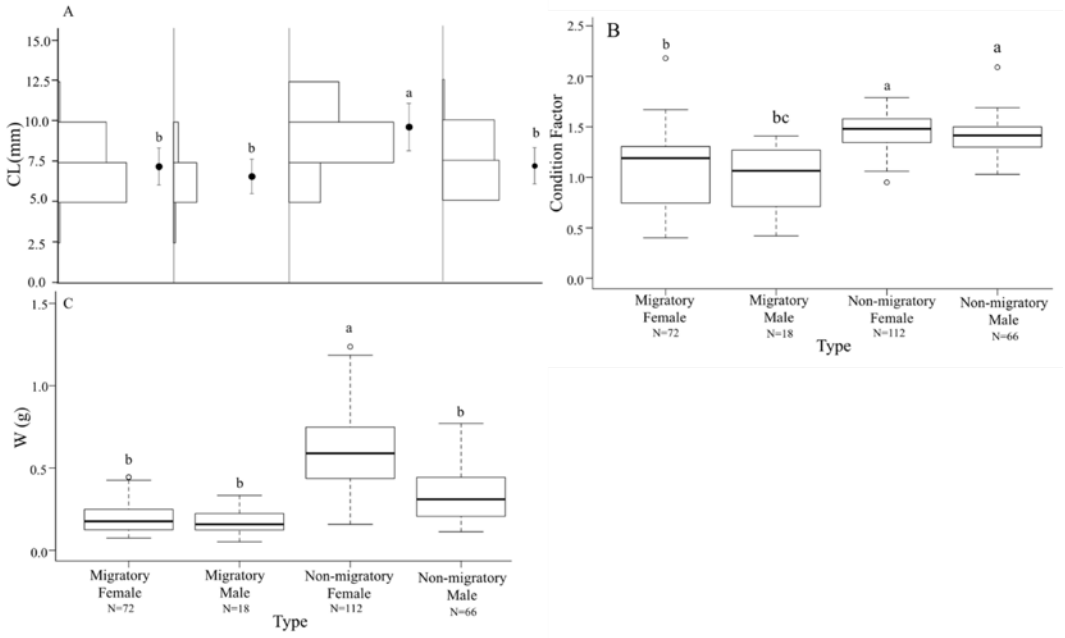

Figure 4.5. Morphological difference between males and females with different behaviors. (A) The difference in carapace length (CL). Dots and bars respectively indicated the means and standard deviations of CL. (B) The difference in and condition factor (CF). (C)The difference in weight (W). Different letters indicate statistically significant differences.

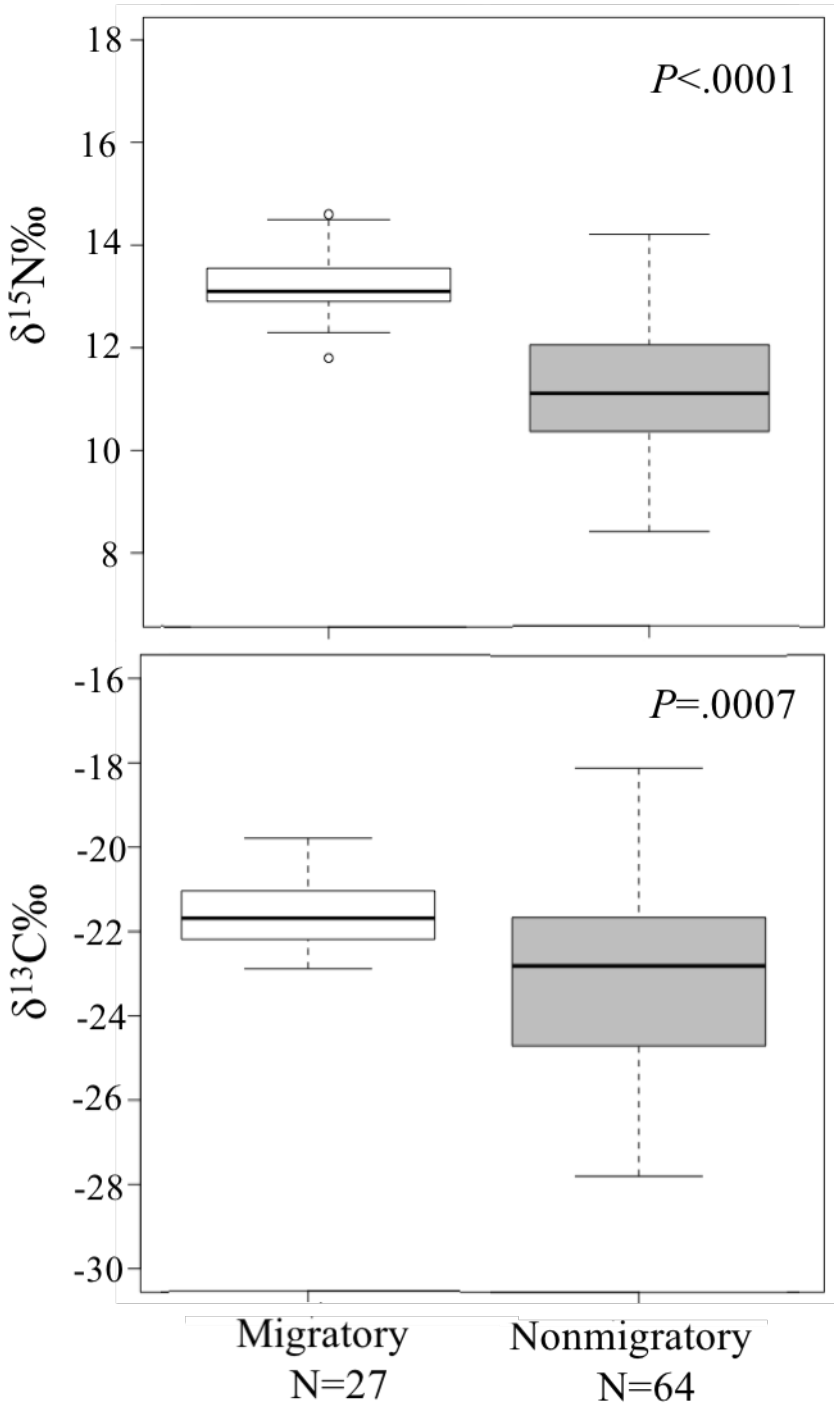

Figure 4.6. The comparison between stable isotope ratio of non-migratory and migratory individuals in winter. A, nitrogen stable isotope ratio and B, carbon stable isotope ratio of *Palaemon paucidens*.

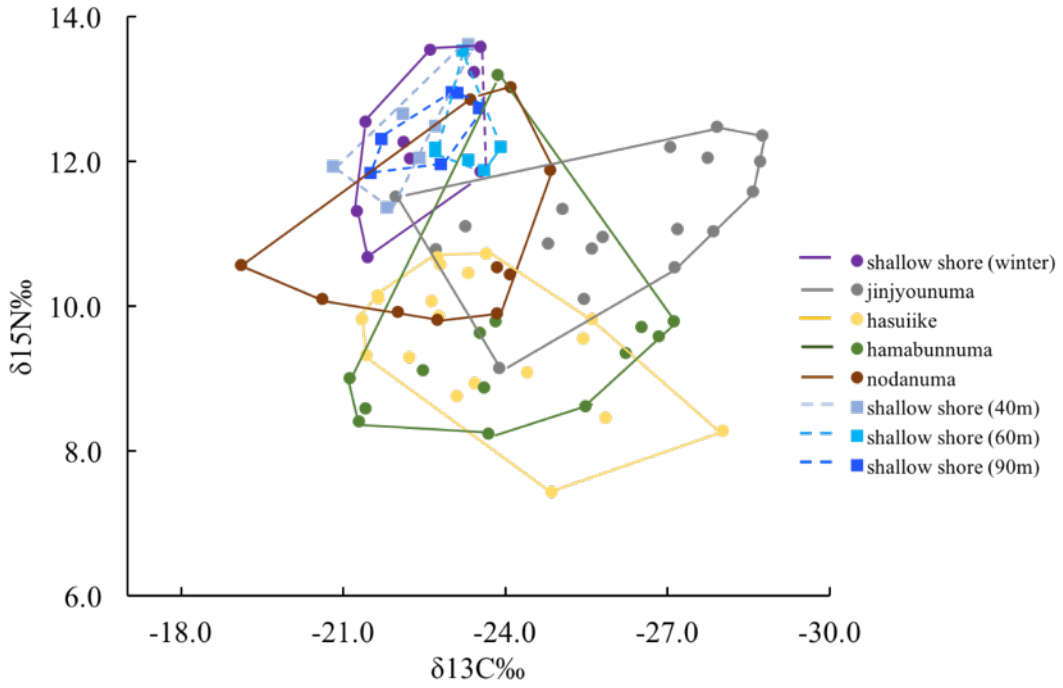

Figure 4.7. The C-N maps of *Palaemon paucidens* in Lake Biwa. The range from sky blue to deep blue within dotted lines indicate migratory individuals that were collected in deep sites in winter; the range from purple to brown within solid lines indicate non-migratory individuals that were collected in shallow sites in winter.

Table 4.1. The information on sampling locations where *Palaemon paucidens* individuals were collected.

Site	Population	Collect date	Latitude	Longitude	Survey tool	N	Mean Ho	Mean He	HWE- *p* Valne
S1	Shiotsu-40mwinter	14/11/2014	35º28’05"	136º10’29"	trawl net	30	0.63	0.83	0.08
S2	Shiotsu-60m-winter	16/01/2015	35º28’05"	136º10’29"	trawl net	26	0.81	0.95	0.08
S3	Shiotsu-90m-winter	24/03/2017	35º22’28"	136º05’24"	trawl net	30	0.68	0.78	0.19
S4	Shiotsu-shallow-winter	24/03/2017	35º26'32"	136º11'21"	basket traps	29	0.62	0.79	0.06
S5	Jinjonuma-inflow-winter	08/12/2016	35º13'36"	136º09'40"	seine traps	10	0.7	0.77	0.34
	Jinjonuma-outflow-winter	08/12/2016	35º13'45"	136º09'30"	seine traps	22	0.70	0.79	0.34
	Jinjonuma-inflow-summer	29/06/2016	35º13'36"	136º09'40"	seine traps	28	0.73	0.79	0.37
S6	Hasuike-inflow-winter	08/12/2016	35º19'14"	136º16'15"	seine traps	15	0.68	0.79	0.35
	Hasuike-outflow-winter	08/12/2016	35º19'14"	136º16'10"	seine traps	34	0.62	0.80	0.38
	Hasuike-inflow-summer	29/06/2016	35º19'14"	136º16'15"	seine traps	30	0.74	0.80	0.49
	Hasuike-outflow-summer	29/06/2016	35º19'14"	136º16'10"	seine traps	29	0.69	0.79	0.24
S7	Kohoku-Nodanuma-inflow-winter	09/12/2016	35º26'59"	136º11'54"	seine traps	16	0.73	0.78	0.49
	Kohoku-Nodanuma-outflow-winter	09/12/2016	35º27'00"	136º11'46"	seine traps	4	0.85	0.92	0.45
	Kohoku-Nodanuma-inflow-summer	30/06/2016	35º26'59"	136º11'54"	seine traps	29	0.72	0.79	0.42
S8	Hamabunnuma-inflow-winter	09/12/2016	35º25'17"	136º02'39"	seine traps	32	0.69	0.80	0.35
	Hamabunnuma-outflow-winter	09/12/2016	35º25'22"	136º02'44"	seine traps	4	0.75	0.83	0.47
	Hamabunnuma-inflow-summer	30/06/2016	35º25'17"	136º02'39"	seine traps	29	0.74	0.79	0.19

S9	Shiotsu-7m-summer	16/09/2015	35º26'56"	136º11'07"	basket traps	28	0.73	0.81	0.3
S10	Shiotsu-10m-summer	04/08/2015	35º26'56"	136º11'07"	basket traps	29	0.76	0.80	0.5
S11	Shiga-shallow-summer	29/07/2016	35º11'59"	135º55'28"	landing net	28	0.74	0.80	0.54
S12	Hirako-Yanagihirako-summer	28/06/2016	35º02'56"	135º55'31"	seine traps	30	0.72	0.79	0.42
S13	Katada-inflow-summer	28/06/2016	35º07'18"	135º55'24"	seine traps	8	0.90	0.94	0.39

Table 4.2. F_{st} (lower triangle) and D_{est} (upper triangle) values between populatoins based on 6 microsatellite loci from 22 locations of *Palaemon paucidens.* Bold representation is statistically significant ($P < 0.05$).

	SW40	**SW60**	**SW90**	**HSOW**	**HSIW**	**JOW**	**JIW**	**KNOW**	**KNIW**	**HMOW**	**HMIW**	**JIS**	**SS10**	**SS8**	**KS**	**HYS**	**HSIS**	**HSOS**	**JIS**	**KNIS**	**HMIS**	**SGSS**
SW40	–	**0.029**	0.024	-0.008	0.024	-0.027	**0.038**	0.041	0.038	0.023	0.04	**0.036**	0.018	-0.008	0.04	0.019	0.017	0.019	0.019	0.003	**0.034**	0.021
SW60	**0.024**	–	0.019	0.016	0.000	-0.006	0.001	0.001	0.04	0.005	0.04	-0.01	0.012	-0.009	0.023	-0.003	0.009	0.014	0.007	-0.007	**0.035**	-0.02
SW90	0.013	0.018	–	0.012	0.009	-0.018	0.006	0.020	0.025	0.009	0.02	-0.01	0.02	0.009	-0.01	-0.004	0.003	0.002	0.026	0.003	0.03	0.016
HSOW	0.016	0.02	0.015	–	-0.011	-0.036	0.029	0.018	0.006	0.002	0.019	0.003	0.006	0.016	0.02	0.002	0.001	0.013	**0.037**	0.018	**0.038**	0.027
HSIW	0.018	0.01	0.021	0.013	–	-0.021	**-0.001**	-0.002	0.003	**0.029**	0.019	0.004	0.018	0.011	0.022	0.02	0.014	0.003	0.018	0.012	**0.023**	0.016
JOW	0.02	0.022	0.018	0.019	0.015	–	0.006	-0.04	0.025	-0.011	0.024	0.001	-0.015	0.003	0.015	-0.003	0.001	**0.002**	-0.012	-0.03	-0.01	**0.031**
JIW	**0.021**	0.012	0.015	**0.019**	0.009	0.019	–	-0.006	0.003	0.01	0.034	-0.01	**0.009**	-0.002	0.019	0.019	0.007	0.016	0.026	0.005	**0.047**	-0.004
KNOW	0.025	0.014	0.026	0.02	0.011	0.018	0.012	–	0.005	0.022	0.02	-0	0.021	0.014	0.018	0.013	0.003	0.016	**0.019**	0.005	0.017	0.017
KNIW	0.052	0.036	0.012	0.043	0.037	0.051	0.036	0.04	–	0.003	0.049	0.023	0.018	0.005	0.03	0.02	0.022	-0.012	0.022	0.002	0.023	0.02
HMOW	**0.018**	0.011	0.01	0.015	**0.011**	0.017	0.01	0.014	0.036	–	0.025	0.012	0.023	0.006	0.026	0.011	0.022	0.013	0.015	0.007	0.06	0.017
HMIW	0.038	0.039	0.023	0.033	0.033	0.038	0.036	0.037	0.063	0.036	–	0.025	0.019	0.02	0.032	0.019	0.017	-0.009	0.02	0.02	0.023	0.02
JIS	**0.021**	0.01	0.014	0.017	0.009	0.021	0.01	0.013	0.033	0.01	0.035	–	-0.01	0.012	0.025	-0.006	-0.009	-0.012	0.012	0.006	0.018	-0.005
SS10	0.016	0.013	0.024	0.014	**0.01**	0.016	0.01	0.015	0.036	**0.011**	0.036	0.009	–	0.020	0.02	-0.003	0.000	0.005	**0.02**	-0.014	0.013	-0.003

SS8	0.011	0.014	0.029	0.014	0.012	0.019	0.012	0.017	0.039	0.011	0.038	0.013	0.012	–	0.026	0.004	-0.005	0.005	0.032	-0.001	**0.048**	-0.005
KS	0.038	0.019	0.01	0.031	0.02	0.028	0.025	0.024	0.042	0.02	0.046	0.022	**0.026**	0.024	–	0.003	0.002	-0.009	0.003	**0.003**	0.033	0.009
HYS	0.021	0.009	0.018	0.016	0.01	0.019	0.012	0.013	0.034	0.01	0.037	0.009	0.01	0.013	0.019	–	-0.012	0.01	0.015	-0.002	0.019	-0.011
HSIS	0.018	0.013	0.011	0.015	0.009	0.018	0.01	0.013	0.039	**0.011**	0.035	0.009	0.008	0.011	0.026	0.009	–	-0.025	0.013	-0.003	0.034	-0.003
HSOS	0.016	0.014	0.011	0.015	0.009	0.019	0.012	0.015	0.041	0.01	0.035	0.008	0.009	0.01	0.025	0.012	0.007	–	0.013	-0.001	0.02	0.018
JIS	0.017	0.012	0.02	**0.019**	0.011	0.017	**0.013**	0.015	0.043	0.01	0.035	0.012	0.011	**0.014**	0.026	0.012	0.01	0.01	–	-0.001	**0.028**	0.011
KNIS	0.017	0.009	0.012	0.017	0.009	0.016	0.01	0.013	0.038	0.009	0.038	0.01	0.007	0.011	0.021	0.009	0.008	0.01	0.009	–	0.012	-0.005
HMIS	**0.019**	**0.015**	**0.017**	**0.018**	**0.011**	0.017	**0.015**	0.014	0.044	**0.015**	0.039	0.012	0.01	**0.016**	**0.028**	**0.012**	**0.013**	0.012	**0.012**	0.01	–	**0.041**
SGSS	0.018	0.01	0.021	**0.018**	0.01	0.021	0.009	0.015	0.033	0.01	0.034	0.01	0.008	0.01	0.023	0.009	0.008	**0.012**	0.01	0.008	**0.013**	–

Table 4.3. Analysis of molecular variance (AMOVA) of *Palaemon paucidens* using 6 microsatellite loci.

Source of variation	**Sum of squares**	**Variance components**	**Percentage variation**	***F*-statistic**	***P***
Two geographical groups (migratory individuals; non-migratory individuals)					
Among groups	3.129	0.00052	0.02	F_{CT} = 0.00022	0.46
Among populations with groups	25.570	0.01189	0.49	F_{SC} = 0.00494	0.32
within populations	1036.644	2.39410	99.48	F_{ST} = 0.00516	0.30

Table 4.4. The result of the sex ratio of non-migratory and migratory individuals.

Coefficients	Estimate	Std. Error	Z value	Pr (>|z|)
(Intercept)	1.457	0.312	4.67	<0.0001***
Migratory behavior (non-migratory/migratory)	-0.925	0.387	-2.39	0.017*

[a]Asterisks show the significant effects of each parameter (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$)

Table 4.5 Summary results of generalized linear mixed models.

(a) The difference in carapace length (CL) between males and females with different behaviors.

Coefficients	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
(Intercept)	6.888	0.376	18.32	<0.0001***
Migratory male	-0.563	0.313	-1.8	0.073
Non-migratory female	2.288	0.472	4.85	<0.0001***
Non-migratory male	0.469	0.48	0.98	0.329

(b) The difference in condition factor (CF) between males and females with different behaviors.

Coefficients	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
(Intercept)	1.02452	0.10603	9.66	<0.0001***
Migratory male	-0.00965	0.05155	-0.19	0.8515
Non-migratory female	0.42648	0.1338	3.19	0.0014 **
Non-migratory male	0.37045	0.13457	2.75	0.0059 **

(c) The difference in weight (W) between males and females with different behaviors.

Coefficients	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
(Intercept)	0.1938	0.0632	3.07	0.0022 **
Migratory male	-0.0218	0.0468	-0.47	0.6419
Non-migratory female	0.4157	0.0795	5.23	<0.0001***
Non-migratory male	0.1481	0.0806	1.84	0.0662

[a]Asterisks show the significant effects of each parameter (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$)

Chapter 5. Habitat selection and migration of the common shrimp, *Palaemon paucidens* in Lake Biwa, Japan – an eDNA-based study

5.1 Introduction

Lake Biwa is the largest freshwater lake in Japan, and the third oldest lake in the world (Nishino & Watanabe, 2000). Approximately 2,400 species of animals, including 66 endemics are present in this Lake (Nishino, 2012). There are four species of shrimp (Nishino, 1978; Nishino & Niwa, 2004), one of which, *Palaemon paucidens,* accounts for most of the biomass, and is an important food source for fish (Harada, 1966; Narita, 2002). It was reported that approximately 1400 tons per year of catch were recorded in the late 1970s, and that the catch of this species has declined significantly from 90 to 40 tons after 2007 (Shiga Prefecture, 2009).

Most *P. paucidens* has been reported to seasonally migrate from shallow waters to offshore bottom sites where they spend winter season; meanwhile, in spring, they migrate from the offshore bottom to shallow waters for reproduction (Harada, 1966; Nishino, 1983). Chapter 2 found that relatively large individuals started to reproduce between May and July, with a peak detected between August

and September. While it has been reported that most individuals move to bottom sites in winter (Harada, 1966; Nishino, 1983), non-migratory individuals overwinter were found in shallow waters (Chapter 3). However, the distribution and abundance of this species has not been investigated with respect to these two different movement types.

Shrimp cages and trawl nets are conventionally used to investigate the movements of *P. paucidens.* However, these tools are unsuitable for detecting small populations, and make for time consuming and labor-intensive monitoring. In recent years, environmental DNA (eDNA) analysis has been widely used for species detection and biodiversity monitoring (Ficetola *et al*., 2008; Thomsen *et al*., 2012). Environmental DNA represents all types of DNA found in the environment, including DNA from organisms in the form of metabolic waste, damaged tissue or sloughed skin cells (Lydolph *et al*., 2005; Hambler *et al*., 2011). Environmental DNA analysis has been applied to macro-organisms (Ficetola *et al*., 2008; Jerde *et al*., 2011; Minamoto *et al*., 2012) and to date,

many eDNA analyses have reported successful monitoring of the presence/absence of rare species (Dejean *et al.*, 2011). In combination with quantitative polymerase chain reaction (qPCR), eDNA concentrations can be used as a proxy for it abundance of crustacean species（Carim *et al.*, 2016; Chapter 3）.

The purpose of this study was to use eDNA to examine the spatial and temporal distribution of *P. paucidens* in Lake Biwa. Water samples were collected from offshore (both from the surface and the bottom), and from the shallow shore of the main lake and its connecting freshwater lagoons. Based on my results, the timing of migration was estimated, and whether the habitat preferences of *P. paucidens* are related to shoreline landscape types was considered.

5.2 Materials and Methods

5.2.1 Study area

Lake Biwa is located on central Honshu Island (35°01′ N, 136°00′ E, Figure 5.1). This lake consists of southern and northern basins. The southern basin is small

(52.5 km^2) and shallow (average depth, 4 m; maximum depth, < 7 m); in contrast, the northern basin is large (617.8 km^2) and deep (average depth, 43 m; maximum depth, 103.6 m) (Okamoto, 1984; Okuda & Kumagai, 1995).

5.2.2 Offshore sampling

To examine the offshore distribution of *P. paucidens* water samples were collected from 41 sites (O1–O41) in the summer (19, 25–28 August, 2016) and in the winter (18, 19, January and 20 February, 2017) (Figure 5.1, Table 5.3). The 1 L samples were collected from the surface and 1 m above the bottom using a bucket and van Dorn sampler, respectively. The distance between each sampling site was 1 km. One mL 10% (mass/volume) benzalkonium chloride solution was added to each sample to prevent DNA degradation (Yamanaka *et al.*, 2017). In summer, some of the water samples (samples from sites O27–O32) were filtered on the survey boat when it was not raining; when rain prevented this, then benzalkonium chloride was added to the samples on return to the laboratory. In

winter, the same volume of benzalkonium chloride was added to all water samples upon collection. Four sites (O18, O20, O22, and O23; Table 5.3) were unsampled in January 2017 because of bad weather. A field blank was used on each sampling day. The field blanks contained 1 L of ultrapure water taken from the laboratory and brought to the field site to check for unintended cross-contamination during sampling and transportation. The lid of the blanks in the field was opened, 1 mL 10% benzalkonium chloride solution was added, and they were brought back to the laboratory. The water samples collected from the surface and the bottom in summer were designated as Summer-Surface and Summer-Bottom samples, respectively; samples collected from the surface and bottom in winter were designated as Winter-Surface and Winter-Bottom samples, respectively.

The water samples were filtered using 47 mm glass-fiber filters (GF/F; GE Healthcare Japan, Tokyo, Japan; nominal pore size = 0.7 μm; cf. Minamoto *et al.* (2016) for selection of filter type for eDNA sampling). After filtration, filters were stored at − 20 °C until DNA extraction.

5.2.3 Shallow shore sampling

One L water samples were collected from 26 shallow shore sites (S01–S26; Figure 5.2; Table 5.4) from spring to winter (23 May, 2 August, and 25 October, 2016 and 6 February, 2017). Water samples were collected from four types of shallow shore sites: sandy beach (S1, S6, S7, S10, S12, S20, S23, S24, and S26), artificial lakeshore (S2, S4, S11, S14, S16, and S22), vegetation (S3, S5, S9, S13, S19, S21, and S25), and reef (S8, S15, and S18). Benzalkonium chloride solution was added to each sample as described above. Each water sample was collected carefully from the surface water to avoid sediment resuspension. The field blanks were adopted as above, and filtration was carried out under the same conditions as described above. Four samples (S23–S26; Table 5.4) in May, 2016 were not collected because of seasonal change in the water level.

5.2.4 Freshwater lagoon sampling

The sampling and DNA extraction methods for lagoon samples slightly differed from those used for the other sample types, because lagoon samples were collected for a separate project. Chapter 3 reported that these differences would not influence the results. Water samples were also collected from 32 freshwater lagoon sites (L1–L32, Figure 5.2; Table 5.5) from autumn to summer on 16 November 2015, 5 February, 6 May, and 5 August 2016. Once collected, the 500 ml samples were filtered on-site as described by Yamanaka *et al*. (2016b) and filtered samples were placed in a cooler box filled with ice packs precooled in ultralow freezers (NEOICE-16/Hard1250). The cooler box temperature was maintained at approximately − 16 °C and samples were stored in the laboratory at – 20 °C. A field blank was sampled at every 10th site, and filtered it as described above.

Samples were collected from offshore and shallow shore sites, all equipment used to collect water was cleaned with bleach solution (diluted household bleach containing 0.1% sodium hypochlorite) for >5 min to remove residual DNA. A

disposable, sealed plastic bag (DP16-TN1000; Yanagi, Nagoya, Japan) was used to collect samples from freshwater lagoon sites. To prevent contamination, the equipment was cleaned with a bleach solution before reuse. The decontaminated equipment was rinsed with ultrapure water. Disposable gloves were used in all procedures to minimize the risk of contamination.

5.2.5 DNA extraction

For offshore and shallow shore samples, DNA extraction from filter samples followed Uchii *et al*. (2016). Two filters were placed in a single Salivate tube (Sarstedt, Nümbrecht, Germany), and the DNeasy Blood and Tissue Kit were used (Qiagen, Hilden, Germany). Environmental DNA of freshwater lagoon samples was extracted according to Miya *et al.* (2015) with a slight modification of the initial lysis step using the following reagent amounts: 200 µL of ultrapure water, 100 µL of Buffer AL, and 20 µL of proteinase K. After DNA extraction, DNA was

eluted from the DNeasy spin column with 100 µL of Buffer AE (Qiagen) and stored at – 20 °C until qPCR analysis.

5.2.6 qPCR quantification of eDNA

To evaluate the amount of eDNA from *P. paucidens*, quantification of the copy number of 16S rRNA genes was performed using real-time TaqMan PCR with the StepOnePlus Real-Time PCR system (Life Technologies, Foster City, CA, USA). Primers and a probe were established in previous study (Chapter 3) that can specifically amplify a 166-bp fragment of 16S rRNA genes of *P. paucidens* (Table 5.1). The specificity of the qPCR assays has been tested (Chapter 3). Quantitative PCRs were conducted with a 20-µL reaction volume in which there were 900 nM of each primer, 125 nM of TaqMan probe, 1 × Environmental Master Mix 2.0 (Life Technologies), 0.1 µL of AmpErase® Uracil N-Glycosylase (Thermo Fisher Scientific) and 5 µL template DNA. A standard dilution series was used containing 3.0×10^{1} to 3.0×10^{4} copies of a linearized plasmid containing

synthesized artificial DNA fragments of 16S rRNA gene sequence (228-bp) (Table 5.1) as quantification standards for all real-time PCR assays. The qPCR conditions were: 2 min at 50 °C followed by 10 min at 95 °C and by 55 cycles of 15 s at 95 °C and 1 min at 60 °C. All PCR runs including samples, quantification standards, and negative controls were performed in triplicate. The average of the three replicates was regarded as the value of the DNA concentration. When a negative detection was obtained for any of the replicates, the DNA concentration of that replicate was assigned a value of zero (Ellison *et al.*, 2006).

5.2.7 Statistical analysis

To evaluate the offshore seasonal distribution of *P. paucidens* eDNA concentration (O1–O41) a generalized linear mixed model (GLMM) with a normal distribution was used. In the model, collection stations are set as explanatory variables, log transformed eDNA copy numbers as response variables, and site IDs as random effects. When the effect of collection stations (Summer-Bottom,

Summer-Surface, Winter-Bottom, Winter-Surface) was significant, tukey multiple comparisons were performed between collection stations.

To examine the distribution of *P. paucidens* eDNA (presence/absence) in the offshore bottom (O1–O41) during summer and winter, a GLMM with a binomial distribution was used. The absence of *P. paucidens* eDNA = 0 and presence of *P. paucidens* eDNA = 1 were assigned and these presence/absence values were set as response variables; depths, sampling season and their interaction as explanatory variables, and site IDs as random effects.

To compare the relationship between water depth and eDNA concentration (abundance) of *P. paucidens* in the offshore bottom during summer and winter, a GLMM with a normal distribution was used. Log of *P. paucidens* eDNA concentration was used for sites where the eDNA was detected. In the model, the log of the eDNA concentration was set as a response variable; depths, sampling season and their interaction were set as explanatory variables, and site IDs as random effects.

In shallow shore sites (S1–S26) a GLMM with a normal distribution was used to evaluate the seasonal distribution of *P. paucidens* eDNA concentration. In this model, the log of eDNA copy numbers served as the response variables; the sampling seasons and the type of sampling location (sandy beach, artificial lakeshore, vegetation, and reef) as explanatory variables, and site IDs as random effects. When the effects of sampling season and shallow shore type were significant, tukey multiple comparisons among seasons and types were performed.

In freshwater lagoon sites (L1–L32) a GLMM with a normal distribution was used to evaluate the seasonal distributions of *P. paucidens* eDNA concentration. In the model, sampling seasons was set as explanatory variables; the log of eDNA copy numbers as response variables, and site IDs as random effects. When the effect of sampling season was significant, tukey multiple comparisons between collection dates were performed.

The GLMM was run using the Automatic Differentiation Model Builder (glmm ADMB) package (Bolker *et al*., 2012; Fournier *et al*., 2012). All analyses were performed using R version 3.3.2 (R Core Team, 2016).

5.3 Results

5.3.1 Quantification of eDNA copy numbers

In all of the PCR runs, R^2 values of calibration curves were > 0.985. The range of slopes was between −3.639 and −3.324, the range of intercepts was between 44.868 and 48.485, and PCR efficiencies were between 88.32 and 97.43%. Based on the calibration curve of each run and the Ct value of each sample, the copy number of the 16S rRNA gene fragment of *P. paucidens* was calculated (Tables 5.3–5.5).

In all the experiments, no eDNA of target species were detected from negative controls, including field blanks and PCR blanks.

5.3.2 Offshore distribution of eDNA

In summer, eDNA of *P. paucidens* was detected in 4 out of the 41 surface sites and 19 out of 41 bottom sites. The eDNA concentrations at positive sites varied from 4.80×10^1 to 3.58×10^2 and 3.00×10^1 to 5.75×10^2 copies/L (Table 5.3). In winter, 0 and 21 out of the 38 sites from the surface and bottom, respectively, were positive for *P. paucidens* eDNA, and the bottom site concentrations varied from 2.50×10^1 to 4.53×10^5 copies/L (Table 4.3). The *P. paucidens* eDNA concentrations differed significantly between surface and bottom sites both in summer and winter (Figure 5.3A, Table 5.2a). Water samples from the bottom in winter had the highest concentrations, whereas, in summer and winter, eDNA was hardly detected in surface samples.

The detection of *P. paucidens* eDNA in the offshore bottom samples showed that the interaction between depth and season was significant (GLMM, p=0.0069; Table 5.2b; Figure 5.3B). In summer, the detection probability of eDNA was

constant within the range of sampling depths (GLMM, p=0.3864). Conversely, in winter, more eDNA was detected in the deep waters.

The concentrations of *P. paucidens* eDNA in the offshore bottom samples also showed a significant interaction between depth and season (GLMM, p=0.0036; Table 5.2c; Figure 5.3C). In summer, eDNA concentrations were not significantly related to sampling depth (GLMM, p=0.065), but eDNA concentrations increased as sampling depth increased in winter.

5.3.3 Shallow shore distribution of eDNA

Shallow shore eDNA was detected in 15 of the 22 sites, in which concentrations varied from 0.80×10^1 to 1.12×10^3 copies/L in spring. In summer, 16 out of the 26 sites were positive, with concentrations ranging from 4.00×10^1 to 3.52×10^5 copies/L. In autumn, 3 of the 26 sites were positive, with concentrations ranging from 2.30×10^1 to 9.50×10^1 copies/L. In winter, 7 out of the 26 sites were positive, with concentrations ranging from 0.90×10^1 to 2.98×10^2 copies/L (Table

5.4). While eDNA concentrations differed significantly between seasons, this was not associated with the type of shallow shore site (Figure 5.4; Table 5.2d). A peak of eDNA concentration was detected in summer, intermediate values in spring, and significantly decreased values in autumn and winter, with the lowest mean concentrations recorded in autumn.

5.3.4 Freshwater lagoon distribution of eDNA

In freshwater lagoons, eDNA was detected in 12 of the 32 sites, with concentrations varying from 1.92×10^2 to 2.21×10^3 copies/L in spring. In summer, 9 of the 32 sites were positive, with concentrations ranging from 2.09×10^2 to 1.72×10^5 copies/L. In autumn, 13 of the 32 sites were positive, with concentrations ranging from 1.30×10^1 to 4.56×10^3 copies/L. In winter, 7 of the 32 sites were positive, with concentrations ranging from 1.06×10^2 to 3.64×10^3 copies/L (Table 5.4). No significant differences were detected between seasons (Figure 5.5; Table 5.2e).

5.4 Discussion

An understanding of distribution and abundance is a prerequisite for conservation of *P. paucidens*. The distribution of *P. paucidens* eDNA in Lake Biwa was tracked and *P. paucidens* eDNA determined by season and site was found. Chapter 3 identified a positive correlation between eDNA concentrations and the abundance of shrimps, and this correlation is assumed to hold for my research. In these results indicate that the spatial and temporal distribution of *P. paucidens* can be visualized, and its abundance inferred, by checking eDNA concentrations. These results will provide important information for the management of this species.

Previous studies highlighted that most individuals inhabit shallow waters in spring and summer, and migrate to offshore bottom sites where they spend autumn and winter (Harada, 1966; Nishino, 1983). However, some individuals have been reported to overwinter in either the shallow shore or the freshwater

lagoons of Lake Biwa (Chapter 3). Environmental DNA concentrations from shallow shore sites and freshwater lagoons in summer were significantly higher than those at offshore bottom sites in the same season (Figure 5.3A, 5.4 and 5.5) which were found. During winter, a high concentration of eDNA from the bottom of the offshore (Figure 5.3A) was detected, which increased significantly with water depth (Figure 5.3C), suggesting the migration to deep offshore in winter. At the same time, relatively high, and low eDNA concentrations were detected in freshwater lagoons and shallow shore sites, respectively (Figures 5.4 and 5.5). The eDNA of *P. paucidens* was rarely observed in samples taken from offshore surface areas during summer and winter (Figure 5.3A). Because *P. paucidens* especially occur in the bottom of lake, sampling water near the bottom may be more accurate for detection. Furthermore, Turner *et al*. (2015) reported that the eDNA concentration of bigheaded Asian carp (*Hypophthalmichthys* spp.) was higher from sediment samples than from water samples. Thus, to study the

benthos, sampling sediment instead of water may also help to increase detection rates.

In this research, high *P. paucidens* eDNA concentrations were found in shallow shore areas in summer (early August), followed by a significant decrease in autumn (mid-October) (Figure 5.4). In addition, low eDNA concentrations were detected at the offshore bottom sites during summer (late August) (Figure 5.3A). Recent spatiotemporal investigations of *P. paucidens'* distribution showed that some individuals occurred in the offshore bottom areas at the end of August (Idomoto & Hatano, 2015). Thus, the changes in eDNA concentrations between summer and autumn reflected migratory behaviors of *P. paucidens*. *Palaemon paucidens* starting migrating from shallow shore sites to the offshore bottom areas between early August and mid-October is estimated. The low *P. paucidens* eDNA concentrations at offshore bottom sites in summer reflected the individuals which moved from the bottom earlier. This suggests that not all individuals migrate to the bottom simultaneously. A previous study reported that *P.*

paucidens zoea are distributed in the thermocline (10–20 m) of Lake Biwa (Harada, 1966). When the zoea die, they would sink to the bottom, and it is possible that the eDNA detected might come from these dead zoea. Jo *et al.* (2017) pointed out that long DNA fragments had a higher decay rate than short fragments, suggesting that long DNA fragments can remove the effects of the carcasses. Thus, in future studies, long DNA fragments can be used to verify whether or not the detected eDNA originated from dead zoea. Alternatively, it is also possible that some individuals permanently occupy the offshore bottom areas of Lake Biwa. However, no individuals have been found at the offshore bottom areas in July (Idomoto & Hatano, 2015). To clarify this point, more intensive (i.e. more frequent and with more sites) sampling and eDNA surveys in offshore bottom areas are needed.

The eDNA concentrations of *P. paucidens* were not significantly different across the four landscape types in the shallow shores shore sites (Figure 5.4).

This suggests that *P. paucidens* does not favor a specific habitat, but that individuals occupied all four shallow shore landscape types.

In addition, the eDNA of *P. paucidens* detected year-round in freshwater lagoons (Table 5.5) was found which suggests that some individuals are non-migratory, remaining in these lagoons all year. Also, eDNA concentrations in freshwater lagoons were found to be higher than in shallow shore sites between autumn and winter, further suggesting that *P. paucidens* (non-migratory individuals) favor lagoon habitats for overwintering. With the exception of one site (see Table 5.3), the eDNA of *P. paucidens* was widely detected in the shallow shore sites and freshwater lagoons of the north basin during winter. Environmental DNA of *P. paucidens* in the samples from freshwater lagoons, were mainly detected from the north basin of Lake Biwa. Because the water flow and wind in freshwater lagoons are slow, and the nutrient content is higher (Hamabata, 1999), thereby making the freshwater lagoon a viable habitat for non-migratory individuals. In a future research, the areas in which the eDNA of *P.*

paucidens was detected during autumn and winter will be focus on and applying DISTLM multivariate regression will help to identify which environmental factors (e.g., waterflow, wind, nutrient content) influence habitat choice.

Organism detection using eDNA is a powerful new tool for conservation and management. However, the probability of detecting eDNA is likely influenced by several processes, including production, degradation, adsorption, and transport (Barnes & Turner, 2015), as well as potential life history factors such as death or reproduction (Kamoroff & Goldberg., 2018; Bylemans *et al*., 2017). In addition, regular monitoring over an extended period of time is necessary to improve the reliability of the results; however, only a few studies have adopted this approach. De Souza *et al*. (2016) found that the detection probability was influenced by the seasonal activity of organisms across warm and cool seasons based on a 2-year-long investigation. Bista *et al*. (2017) examined temporal shifts in the biodiversity of Chironomidae at regular and frequent intervals over a 1-year period. My study partly clarified the migration ecology of this species despite

being a single-year study. With continuous monitoring, a more detailed understanding of this species through longer-term surveys and consideration of various environmental factors or life history factors are expected to gain.

Previous studies suggested that *P. paucidens* inhabits the offshore bottom in winter and the plant area (vegetation) for reproduction in spring and summer (Harada, 1966; Nishino, 1983). However, eDNA was detected from not only the offshore bottom, but also from lagoons and shallow shore in winter. In addition, the eDNA was detected regardless of the habitat type of shallow shore sites. These findings update information of the habitat of this species. By further expanding the protection range and analyzing the reasons for the reduction in the number of individuals, future studies will contribute valuable information to guide the conservation and sustainable use of this economically important species.

Overall, in this study, eDNA analysis was used to monitor the spatial and temporal distribution of *P. paucidens* in Lake Biwa. In addition, comparing

changes in eDNA concentration at different sites allowed us to speculate on the timing of local migrations, and habitat selection. A firm belief is hold that eDNA analysis is an effective tool improving upon conventional survey methods. This technology not only allows us to evaluate how organisms are distributed, it can also provide more detailed ecological information; for example, timing of local movements, as in this study. This technology also has wide application across a range of species.

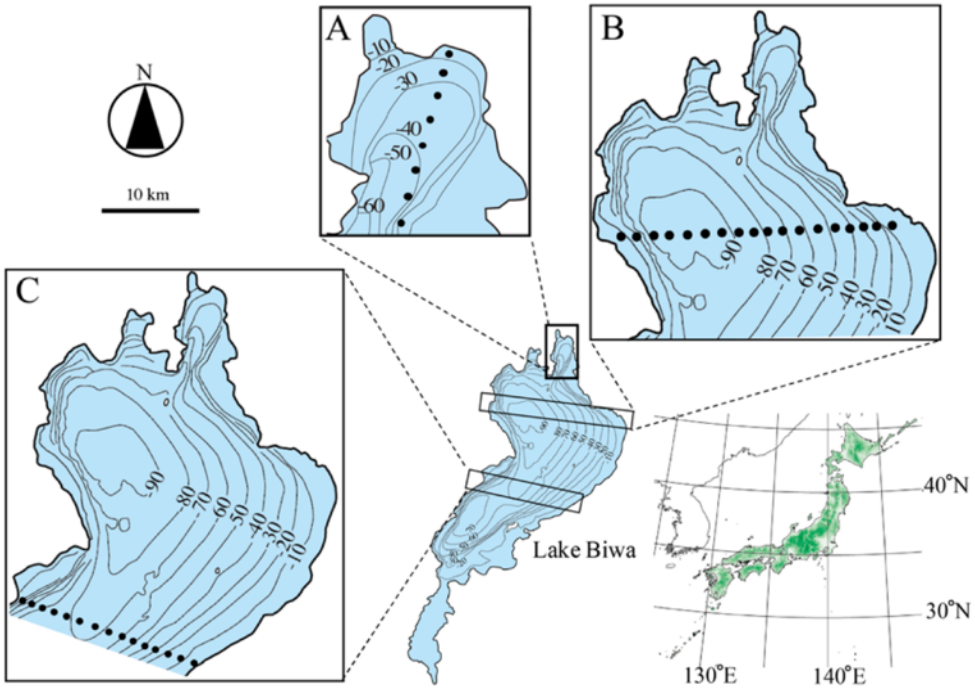

Figure 5.1. Map of the study sites for offshore samples. The water samples were collected from 8 sites from north to south in panel (A) (O1−O8), from 18 sites from west to east in panel (B) (O9−O26), and from 15 sites from west to east in panel (C) (O27−O41). The map of Lake Biwa was modified from Ikeda & Hatano (2015).

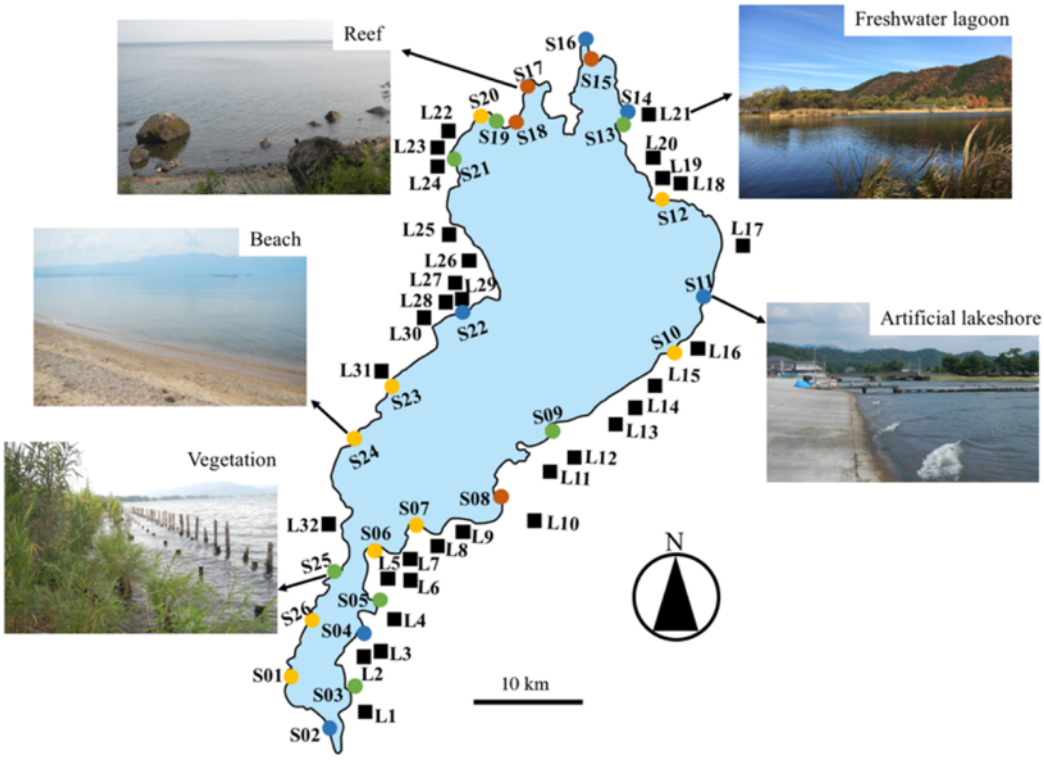

Figure 5.2. Map of the study sites for lakeshore and lagoon sites. Black boxes show the sampling sites within the freshwater lagoon. Circles show the sampling sites along the shallow shore sites. Yellow, blue, green, and brown denotes sandy beach, artificial lakeshore, vegetation, and reef, respectively.

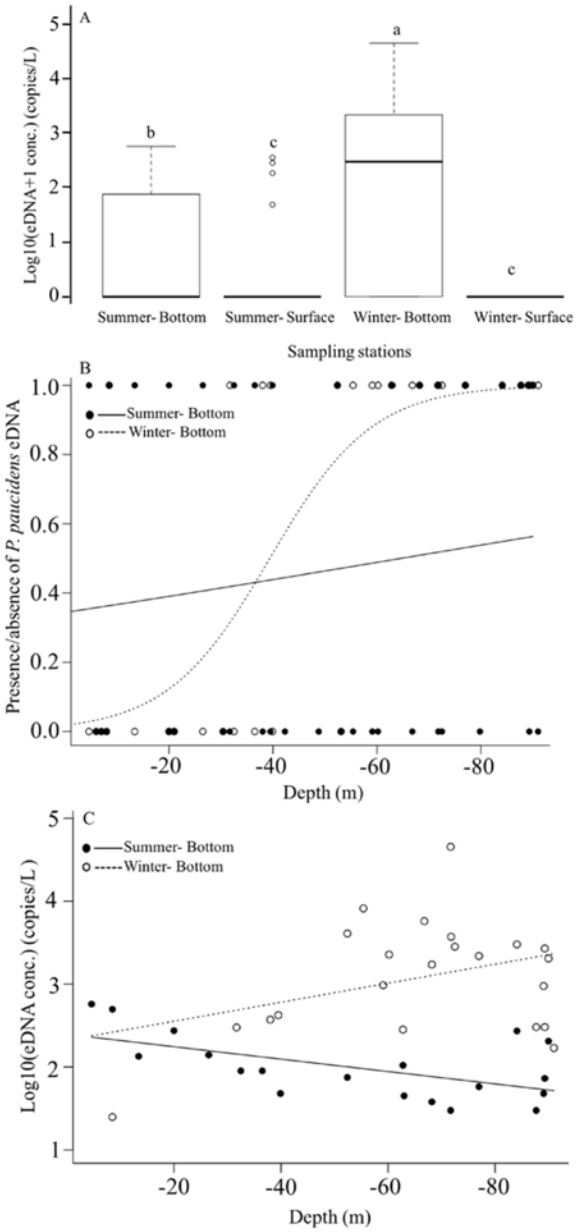

Figure 5.3. *Palaemon paucidens* eDNA concentration offshore. (A) The spatial and temporal variation of *P. paucidens* eDNA concentration offshore. Different letters indicate statistically significant differences. (B) The distribution of *P. paucidens* eDNA; offshore bottom (solid circle, solid line summer; open circle, dashed line winter). In this graph, we assigned presence and absence of *P. paucidens* DNA as 1 and 0, respectively. (C) *Palaemon paucidens* eDNA concentration relative to water depth (solid circle, solid line summer; open circle, dashed line winter). Only the positive results of *P. paucidens* eDNA were plotted.

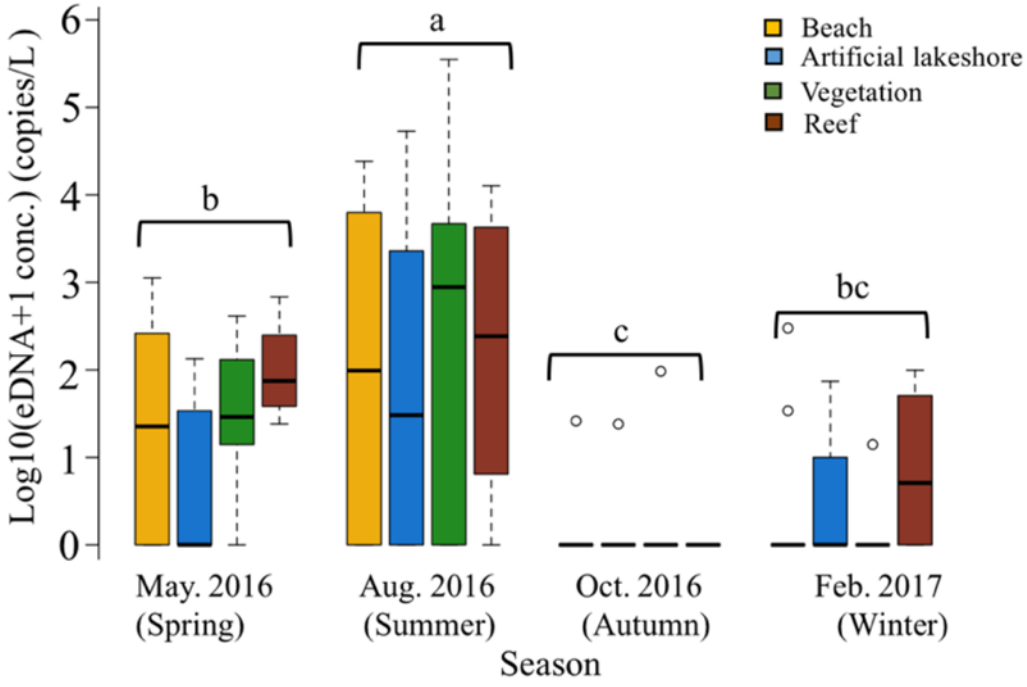

Figure 5.4. Spatial and temporal variation of *Palaemon paucidens* eDNA concentration in the shallow shore sites in Lake Biwa. Yellow, blue, green, and brown denote sandy beach, artificial lakeshore, vegetation, and reef, respectively. Different letters indicate statistically significant differences between seasons. There was no significant difference between types of shores.

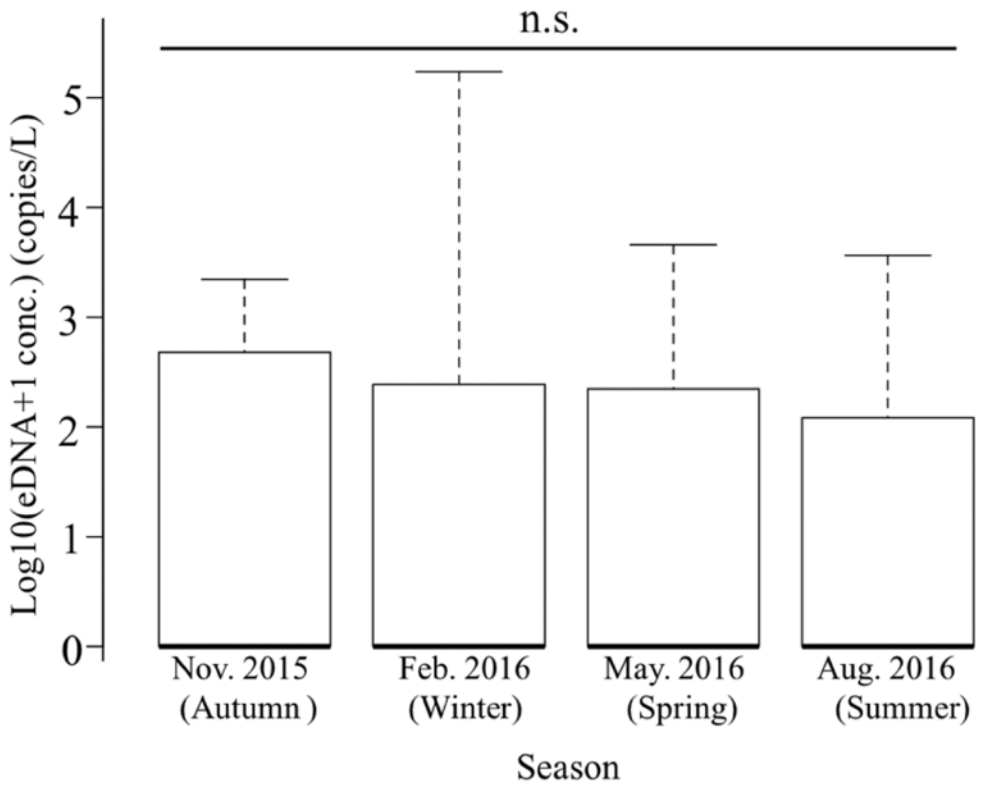

Figure 5.5. Spatial and temporal variation of *Palaemon paucidens* eDNA concentration from freshwater lagoons in Lake Biwa. There was no significant difference between collection seasons (n= 32).

Table 5.1. Primers, probe and DNA sequences within the plasmid used in my study.

Name	**Sequence (5'- 3')**
Palaemon paucidens forward primer	AAGTCTAACCTGCCCACTGAGTTA
Palaemon paucidens reverse primer	TTTAAGCCTTTTCACTTAAAGGTCA
Palaemon paucidens probe	FAM- ATGAGGGAAAAACTG-NFQ-MGB
DNA sequences within the plasmid	GAATTCACAT GTCTATATAG ATTCTTAATA TAAGTCTAA CCTGCCACT GAGTTATTAA AGGGCTGCGG TAATTTGACC GTGCAAAGGT AGCATAATCA GTAGTCTTTT AATTGAAGGC TTGAATGAAC GGTTGGATGA GGGAAAAACT GTCTCTCCTA TAAATTGAAA TTTGACCTTT AAGTGAAAAG GCTTAAATTA ACTAAGGGGA CGATAAGACC CTAAGCTT

Table 5.2. Summary results of generalized linear mixed models.

(a) Offshore seasonal distribution of *P. paucidens* eDNA concentration

Coefficients	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
(Intercept)	0.746	0.135	5.53	<0.0001 ***
Summer-Surface	-0.566	0.191	-2.97	0.003 **
Winter-Bottom	0.851	0.196	4.35	<0.0001 ***
Winter-Surface	-0.746	0.196	-3.81	<0.0001 ***

(b) Seasonal distribution of *P. paucidens* eDNA; offshore bottom

Coefficients	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
(Intercept)	-0.646	0.66	-0.98	0.3281
Depth	0.01	0.012	0.87	0.3864
Winter	-3.247	1.474	-2.2	0.0276*
Depth : Winter	0.091	0.034	2.7	0.0069**

(c) Relationship between water depth and *P. paucidens* eDNA concentration, and between sampling seasons for offshore sites

Coefficients	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
(Intercept)	2.395	0.2468	9.71	<0.0001 ***
Depth	-0.007	0.004	-1.84	0.065
Winter	-0.071	0.431	-0.17	0.868
Depth : Winter	0.019	0.007	2.91	0.0036 **

(d) Seasonal distribution of *P. paucidens* eDNA concentration; shallow shore sites

Coefficients	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
(Intercept)	2.095	0.615	3.41	0.0007 ***
Artificial lakeshore	-0.331	0.717	-0.46	0.644
Vegetation	0.466	0.69	0.68	0.499
Reef	1.671	0.827	2.02	0.053

Summer	1.895	0.615	3.08	0.002 **
Autumn	-2.078	0.615	-3.38	0.0007 ***
Winter	-1.482	0.615	-2.41	0.016 *

(e) Seasonal distribution of *P. paucidens* eDNA concentration; freshwater lagoon

Coefficients	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
(Intercept)	1.0554	0.2432	4.34	<0.0001 ***
Winter	-0.1731	0.2759	-0.63	0.53
Spring	-0.0389	0.2759	-0.14	0.89
Summer	-0.2393	0.2759	-0.87	0.39

[a]Asterisks show the significant effects of each parameter (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$)

Table 5.3. Location details and quantitative real-time PCR (qPCR) from water samples collected from the surface and the bottom in summer (Summer-Surface and Summer-Bottom) and winter (Winter-Surface and Winter-Bottom) in Lake Biwa. Environmental DNA (eDNA) concentration was assessed using qPCR.

ID	Date		Latitude	Longitude	Depth (m)	eDNA concentration (copies/L)			
	Summer	Winter				Summer-Surface	Summer-Bottom	Winter-Surface	Winter-Bottom
O1	20160827	20170119	35°30’12.46"	136°10’56.06"	8	0	0	0	0
O2	20160827	20170119	35°29’41.82"	136°10’49.692"	26.5	0	140	0	0
O3	20160827	20170119	35°29’8.736"	136°10’42.420"	32.5	0	90	0	0
O4	20160827	20170119	35°28’37.92"	136°10’36.156"	36.5	0	90	0	0
O5	20160827	20170119	35°28’5.088"	136°10’29.46"	39.5	0	0	0	423
O6	20160827	20170119	35°27’33.120"	136°10’22.656"	38	0	0	0	373
O7	20160827	20170119	35°27’0.648"	136°10’16.14"	20	185	275	0	0
O8	20160827	20170119	35°26’29.184"	136°10’9.048"	8.5	0	498	0	25
O9	20160826	20170118	35°22’30.936"	136°3’25.524"	7.0	0	0	0	0
O10	20160826	20170118	35°22’29.676"	136°4’5.376"	77.0	0	58	0	2185
O11	20160826	20170118	35°22’28.920"	136°4’44.904"	90.0	0	205	0	2040
O12	20160826	20170118	35°22’28.164"	136°5’24.468"	91.0	0	0	0	170
O13	20160826	20170118	35°22’27.156"	136°6’3.708"	89.3	0	0	0	2695
O14	20160826	20170118	35°22’26.652"	136°6’43.56"	89.1	48	48	0	948
O15	20160826	20170118	35°22’25.644"	136°7’23.124"	89.3	0	73	0	303
O16	20160826	20170118	35°22’24.888"	136°8’2.364"	87.7	0	30	0	303

O17	20160826	20170118	35°22’26.4"	136°8’42,54"	84.1	0	273	0	3020
O18	20160827	-	35°22’27.660"	136°9’20.844"	79.8	0	0	-	-
O19	20160827	20170118	35°22’28.668"	136°10’0.408"	71.7	0	30	0	45303
O20	20160827	-	35°22’29.676"	136°10’39.936"	63.0	0	45	-	-
O21	20160827	20170119	35°22’31.188"	136°11’20.724"	55.4	0	0	0	8188
O22	20160827	-	35°22'33.708"	136°12’0.288"	48.8	0	0	-	-
O23	20160827	-	35°22’34.716"	136°12’40.752"	42.3	0	0	-	-
O24	20160828	20170119	35°22’36.228"	136°13’20.316"	30.4	0	0	0	0
O25	20160828	20170119	35°22’36.984"	136°13’59.232"	21.0	0	0	0	0
O26	20160828	20170119	35°22’39.252"	136°14’39.408"	6.0	0	0	0	0
O27	20160825	20170119	35°16’33.204"	136°1’3.36"	21.0	0	0	0	0
O28	20160825	20170119	35°16’24.888"	136°1’41.988"	52.4	0	75	0	4073
O29	20160825	20170220	35°16’15.564"	136°2’20.328"	59.1	0	0	0	973
O30	20160825	20170220	35°16’6.96"	136°2’58.632"	62.8	0	105	0	283
O31	20160825	20170220	35°15’58.14"	136°3’36.000"	68.2	0	38	0	1723
O32	20160825	20170220	35°15’49.32"	136°4’13.728"	72.5	0	0	0	2830
O33	20160819	20170220	35°15’40.212"	136°4’52.032"	71.8	0	0	0	3723
O34	20160819	20170220	35°15’31.644"	136°5’30.948"	66.8	0	0	0	5750
O35	20160819	20170220	35°15’22.068"	136°6’8.352"	60.2	0	0	0	2273
O36	20160819	20170220	35°15’13.716"	136°6’47.268"	53.1	0	0	0	0
O37	20160819	20170220	35°15’4.644"	136°7’25.284"	39.9	0	48	0	0
O38	20160819	20170220	35°14’55.824"	136°8’2.364"	31.7	0	0	0	300
O39	20160819	20170220	35°14’46.5"	136°8’40.992"	20.0	0	0	0	0
O40	20160819	20170220	35°14’37.896"	136°9’19.296"	13.4	358	135	0	0
O41	20160819	20170220	35°14’28.824"	136°9’57.312"	4.6	285	575	0	0

Table 5.4. Location details and quantitative real-time PCR (qPCR) for water samples collected at Lake Biwa shallow shore sites. Environmental DNA (eDNA) concentration was assessed using qPCR.

Site	Type	Latitude	Longitude	eDNA concentration (copies/L)			
				20160523	20160802	20161025	20170206
S1	Sandy beach	35°01′43.8″	135°52′04.6″	0	0	0	0
S2	Artificial Lakeshore	34°59′24.8″	135°53′46.5″	0	0	0	0
S3	Vegetation	35°01′38.4″	135°55′00.8″	13	0	0	0
S4	Artificial Lakeshore	35°03′51.1″	135°55′49.2″	0	0	0	0
S5	Vegetation	35°05′10.5″	135°56′46.5″	0	0	0	0
S6	Sandy beach	35°07′19.8″	135°56′35.8″	8	0	0	0
S7	Sandy beach	35°08′27.9″	135°59′01.2″	0	95	25	0
S8	Reef	35°09′47.0″	136°03′30.9″	90	1429	0	25
S9	Vegetation	35°12′37.4″	136°06′35.7″	410	17720	95	0
S10	Sandy beach	35°16′01.1″	136°13′13.9″	55	427	0	0
S11	Artificial Lakeshore	35°17′45.6″	136°15′19.1″	133	53299	0	0
S12	Sandy beach	35°23'13.92"	136°13'30.68	1120	97	0	298
S13	Vegetation	35°26′39.2″	136°11′22.0″	130	879	0	0
S14	Artificial Lakeshore	35°27′24.0″	136°11′54.8″	0	2279	0	73

S15	Reef	35°29′57.9″	136°10′27.2″	23	0	0	0
S16	Artificial Lakeshore	35°30′52.1″	136°09′49.4″	33	916	23	9
S17	Reef	35°28′50.1″	136°06′48.7″	60	12645	0	0
S18	Reef	35°27′04.2″	136°05′57.3″	680	40	0	98
S19	Vegetation	35°26′51.5″	136°05′06.2″	43	1223	0	0
S20	Sandy beach	35°27′37.2″	136°04′04.4″	260	6260	0	33
S21	Vegetation	35°25′46.3″	136°02′30.0″	18	0	0	0
S22	Artificial Lakeshore	35°17′8.24"	136°01′3″	0	0	0	0
S23	Sandy beach	35°14′37.2″	135°57′53.2″	-	0	0	0
S24	Sandy beach	34°44′00.1″	135°14′01.3″	-	24083	0	0
S25	Vegetation	35°6'14.8″	135°54'56.0″	-	352378	0	13
S26	Sandy beach	35°3'57.708"	135°53'7.08″	-	21190	0	0

Table 5.5. Location details and quantitative real-time PCR (qPCR) for water samples collected at Lake Biwa freshwater lagoons. The qPCR results show the number of positives in the three replicates. Environmental DNA (eDNA) concentration was assessed using qPCR.

Site	Location	Latitude	Longitude	eDNA concentration (copies/L)			
				20151116	20160205	20160605	20160805
L1	Tonotagawa-naiko	34º59'43"	135º54'36"	0	0	0	0
L2	Yanagihirako	35º02'58"	135º55'34"	0	0	0	0
L3	Hirako	35º03'00"	135º55'12"	0	0	0	0
L4	Shinanaka-naiko	35º03'31"	135º56'51"	0	0	0	0
L5	Erinohama	35º05'02"	135º56'52"	0	0	0	0
L6	Konohama-naiko	35º05'33"	135º56'23"	0	0	0	0
L7	Yasu River	35º07'08"	135º57'50"	0	0	0	0
L8	Yasu River	35º07'29"	135º59'30"	0	466	0	0
L9	Yasu River	35º08'33"	135º03'13"	0	0	521	0
L10	Kitazawanuma	35º08'28"	136º00'20"	0	0	0	0
L11	Kitanoshosawa	35º08'54"	136º05'34"	476	0	0	0
L12	Nishinoko	35º09'48"	136º06'20"	0	0	0	0
L13	Iba-naiko	35º11'17"	136º08'11"	1180	1363	0	138
L14	Jinjonuma	35º13'44"	136º09'28"	2212	0	3425	0
L15	Sonenuma	35º14'36"	136º11'35"	0	0	4560	106

L16	Hikone-Nodanuma	35º14'58"	136º12'36"	0	282	1677	1070
L17	Hasuike	35º19'09"	136º16'09"	1479	5920	215	522
L18	Hosoe-naiko	35º23'16"	136º14'36"	482	306	226	803
L19	Minamiura-naiko	35º24'41"	136º12'33"	0	0	491	1137
L20	Hayasaki-naiko	35º25'03"	136º12'14"	0	0	43	0
L21	Kohoku-Nodanuma	35º27'05"	136º11'48"	0	172248	13	2300
L22	Nukigawa-naiko (north)	35º25'56"	136º02'21"	892	0	0	0
L23	Nukigawa-naiko (south)	35º25'46"	136º02'25"	0	0	0	0
L24	Hamabunnuma	35º25'22"	136º02'42"	544	209	71	3639
L25	Harieokawa	35º21'54"	136º03'16"	1025	0	0	0
L26	Suganuma	35º20'56"	136º04'07"	192	0	0	0
L27	Ekainuma	35º19'16"	136º03'32"	0	0	115	0
L28	Gotandanuma	35º19'09"	136º02'45"	286	2467	0	0
L29	Matsunoki-naiko	35º18'47"	136º03'14"	286	583	344	2042
L30	Otomegaike	35º17'31"	136º00'52"	834	0	588	0
L31	Oumimaikonuma	35º14'23"	135º57'57"	0	0	0	0
L32	Katata-naiko	35º06'53"	135º55'21"	0	0	0	0

Chapter 6. General discussion

This study has provided novel knowledge of *P. paucidens* ecology in Lake Biwa, explained the behavior of migratory individuals moving to the bottom of the lake in winter, and confirmed the existence of non-migratory individuals. Several chemical experiments were used to compare the nutritional condition of *P. paucidens* moving to the bottom of the lake and staying in the shallow shores. The results showed the nutritional condition of *P. paucidens* remained constant throughout the year, which meant they moved to the bottom of the lake to feed. Such a phenomenon explains the mystery of the migratory individuals moving to the deep water in the autumn. Then, *P. paucidens* eDNA was detected in the shallow shores and freshwater lagoons during the autumn and winter, which indicates the existence of the non-migratory individuals. Moreover, it was clarified that there was no difference in genetic structure between migratory and non-migratory individuals. There was no significant difference in size between the non-migratory males and the migratory males, and the non-migratory females were larger than migratory females, non-migratory males and migratory males.

Furthermore, the results showed significant differences of the sex ratios between migratory and non-migratory individuals, in which the majority was female in migratory individuals, while it was male in non-migratory individuals. At the same time, different concentrations of *P. paucidens* eDNA was detected provide a basis for grasping this species' spatial and temporal distribution in Lake Biwa. Moreover, the season, during which individuals move from the shallow shores to the bottom of the lake can be determined according to the changes in the eDNA concentration found in the shallow shores during summer and autumn.

In Chapter 2, changes in the body size, lipid content, nucleic acid ratio, and stable isotopic ratio were analyzed between individuals before and after migration to the deep water for the purpose of better understanding the species' ecology and migratory behavior. The experimental results in this chapter suggested that, *P. paucidens* in Lake Biwa migrate to the deep water in winter is not to hibernate as previous studies have discussed. Instead, there are sufficient food sources in the deep water (i.e., *Jesogammarus annandalei* and Oligochaete). Thus, migrating to

the deep water can prevent starvation. At the same time, *P. paucidens* can accumulate energy by such a migration pattern; thereby preparing for subsequent migration (from bottom to the lakeshore), and migration to the lakeshore for spawning purposes. Such migratory behavior would be a beneficial strategy that ensures this species can survive and reproduce.

In Chapter 3, the existence of non-migratory individuals in winter in Lake Biwa was confirmed by the eDNA analysis method. The results of this study suggested that, other than the seasonally migrating individuals, there are non-migratory ones in the Lake Biwa which inhabit the shallow shores during winter. In other words, *P. paucidens* in the Lake Biwa exhibit at least two different behavioral patterns.

In Chapter 4, the genetic structure and morphological characteristics which included sex, body size and condition factor of migratory and non-migratory individuals were analyzed. In addition, by means of the analysis of carbon and nitrogen stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$), the food source and trophic level

of the polymorphism migratory behaviors exhibited by *P. paucidens* have been estimated. While individuals belonging to this species were genetically indistinguishable regardless of their migratory behaviors, the non-migratory female individuals were larger than their migratory counterparts in terms of body size and condition factor. The stable isotope ratios of the migratory individuals had no significant differences between sampling sites, thereby suggesting the presence of identical food sources in the deep water. On the contrary, stable isotope ratios of the non-migratory individuals had significant differences between sampling sites, which suggests that the food sources of non-migratory individuals would vary according to their location in the shallow shores.

In Chapter 5, the timing of migration from shallow shores to deep water, spatial and temporal distribution, as well as habitat preferences of *P. paucidens* in Lake Biwa were estimated by tracking their eDNA concentration. Furthermore, the eDNA concentration in summer in the shallow shores and freshwater lagoons turned out to be significantly higher than that in offshore bottom. In contrast, a

high concentration of eDNA was detected in offshore depth in excess of 50 m in winter. The eDNA concentration in the offshore bottom was the highest, followed by the freshwater lagoon and the shallow shores in winter. The changes in eDNA concentration from the shallow shores to offshore bottom show that this species migrates between early August and mid-October. This species does not favor a specific habitat, as the individuals occupy all four shallow shore landscape types.

The life histories of both migratory and non-migratory individuals is rearranged and shown according to the results of this study and the previous research reported (Figure 6.1). The life history of this species, which inhabit Lake Biwa was summarized as follows. The breeding period of the migratory individuals in Lake Biwa lasts from May to September, peaking between August and September. It takes approximately 24 days for new individuals to progress from egg to nauplius, zoea, megalopa and finally to the juvenile stage of its lifecycle (Shokita, 1975). During the period in which individuals developed from nauplius to megalopa, they appeared in the thermocline which occur in summer

within the water depth of about 10-20m (Harada, 1966), before moving to the shallow shores at the juvenile stage. During the breeding period, although most adults die-off after spawning, a small proportion of reproductive female individuals can survive. From early August to mid-October, post-spawning female individuals and new individuals migrate from shallow shores to offshore bottom. Most individuals occur in areas with water depths of more than 50m in winter. *Palaemon paucidens* migrate to the bottom of the lake to feeding sites where they are able to store energy in preparation for later breeding. However, from November to April of the next year, post-spawning females with large size disappeared, and the proportion of post-spawning females decreased from 40% to 19%; potentially as a result of death or their movement to other places when the sample was being collected. During the reproduction season in August and September, the proportion of males dramatically decreased. This result showed the death of the male individuals. These results suggested that most females generally have a life span of one year,

but there are a small number of females with a life span of more than one year. On the other hand, males have a life span of one year.

As for about non-migratory individuals, the results of eDNA concentration showed that a few non-migratory individuals inhabit the shallow shores and the freshwater lagoon during autumn to winter, which is consistent with the previous study (Harada, 1966). When non-migratory individuals live with migratory individuals during spring to summer, it is not possible to distinguish them correctly based on morphological or genetic features. Developing experimental methods that can identify non-migratory individuals is important for understanding their ecology in the future.

The causes behind the movement of animals may be various (Morita & Morita, 2007). Tsukamoto & Kajihara (1987) argues that limited food, increasing density and other environmental stress could drive animals to migrate and escape. According to a study on the movement of trout, it is mentioned that the individuals which lose in the group will go down into the sea and grow up in the

sea through forages (Morita *et al*., 2004). While *P. paucidens* populations that exhibit different migratory behaviors are genetically indistinguishable (Chapter 4), there are no post-spawning females among the non-migratory individuals collected from the freshwater lagoons. Furthermore, the non-migratory females collected are larger than migratory individuals. According to the results above, whether the *P. paucidens* are migratory or non-migratory is presumed on their growth, nutritional or reproductive status. Such behavior in this species can be regarded as a conditional strategy, as has been observed in salmon (Kaeriyama, 1996; Aubin-Horth & Dodson, 2004).

In July, the proportion of males to females was equal, while the sex ratio was biased to females in other months. The same phenomenon has been reported in other species that the total sex ratio of the Mar del Plata harbour population of *Palaemon macrodactylus* was biased to females (Vázquez *et al*., 2012). Among life-history traits, the sex ratio is closely related to a species' reproductive strategies, with natural selection commonly favoring a 1:1 ratio (Fisher, 1958). A

sex ratio different from 1:1 could be attributed to one of five interpretations: longevity factor, differential migration, differential mortality, differential growth rates or sex reversal (Wenner, 1972). In this study, the reason why *P. paucidens* in Lake Biwa was biased to females would be due to the death of most males after mating (life span is one year) and due to survive of some females after spawning (life span is more than one year). In addition, comparing the results of isotopes in the shallow shores in winter, the food nutrition level of migratory individuals in the offshore bottom in winter is obviously higher than that in the shallow shores in winter, and the offshore bottom shows the same food. Among the migratory individuals in winter, approximately 80% of them were females and the rest of them were males. Therefore, it is possible that the choice of moving to the offshore bottom in winter is considered a beneficial strategy for most of females that ensures them survive and reproduce.

It is not yet fully understood why, in a population consisting of non-migratory individuals, there are no post-spawning females. It is possible that

shrimps' abdomen characteristics disappear after shelling. Studies on trout have indicated that the various reproductive strategies for residual male individuals (e.g. sneaky; fighter). Individuals that adopt a sneaky strategy also showed the common feature of precocious puberty (Dalley *et al.*, 1983; Bohlin *et al.*, 1986; Thorpe, 1986). The ayu-fish (*Plecoglossus altivelis*) which also inhabit Lake Biwa is divided into o-ayu and ko-ayu according to their different body size. It can be observed that even ko-ayu inhabiting Lake Biwa are more sexual precocity than o-ayu (Azuma, 1981). While adopting a sneak strategy is not very significant for shrimps, individuals with precocious characteristics can reproduce immediately during the breeding season. Furthermore, individuals born earlier grow up faster than those born later. It can be assumed that non-migratory female individuals in Lake Biwa have also adopted the strategy of early maturity in an effort to improve their offspring's survival rate or growth rate. In order to prove this possibility, the gonadal state of the shrimp collected from the bottom of the

lake and those collected from the shallow shore during winter should be compared.

According to the aforementioned results, the different behaviors exhibited by the possibility of certain conditions (i.e. individual size and nutritional status) of *P. paucidens*. Several organisms exhibit different morphological characteristics at different stages (age) (e.g. amphibian, insect; Houck, 1977, Denno & Dingle, 1981). In this research, it was found that a small number of female individuals live longer surpassing one year. Shrimps' abdominal characteristics disappear because of shelling, and the possibility of individuals moving from the bottom of the lake to the shore in following year. The results of this study cannot completely prove the different behavior patterns and ecological characteristics of *P. paucidens* which are caused by differences in growth stage. Further research is necessary to confirm whether migratory and non-migratory individuals are determined by the growth stage.

Palaemon paucidens is a species closely related to the people's lives in Shiga Prefecture, as it is an economically important species. The protection of this species also works in protecting Shiga's traditional culture. Not only Prefectural Fisheries Division and local fishermen, but also the citizens of the prefecture should make a concerted effort to protect the species in the future. The ecological knowledge of *P. paucidens* living in Lake Biwa was introduced to the citizens in the form of "Science Cafe" in Otsu City in Shiga Prefecture in June 2019, and the ideas of how to protect this species were exchanged in order to allow the public to better understand this species. A survey done in "Science Cafe" showed that the younger generations (middle school students) had great interest in the ecology of this species. It is expected that more and more citizens will be interested in this species and getting more understanding about this species through regular scientific lectures held in the form of "Science Cafe" or other way.

Meanwhile, workshops of eDNA technology held on a regular basis may also help the citizens understand about this species. In this way, more and more

citizens can actually take part in the investigation of this species by means of eDNA analysis. Through activities by eDNA workshops, the citizens can not only learn the knowledge about these species, but also participate in related experiments. The citizens are also expected to have better understanding about this species and become conscious of the connection between the species and themselves. The citizen's awareness of the preservation of this species are thus expected to be raised.

In this study, several techniques were used to comprehend the life history and the distribution of *P. paucidens* in the whole Lake Biwa. These results provide vital evidence for future management and conservation of this species. Those methods of this study should be effective in studying the life history and the distribution of other organisms. Apart from it, currently, no effective method has been developed to distinguish the migratory and non-migratory individuals in spring and summer when they mixed together. In the future, developing experimental methods that can identify migratory and non-migratory individuals

at the same time will play an important role in the ecological research of this species.

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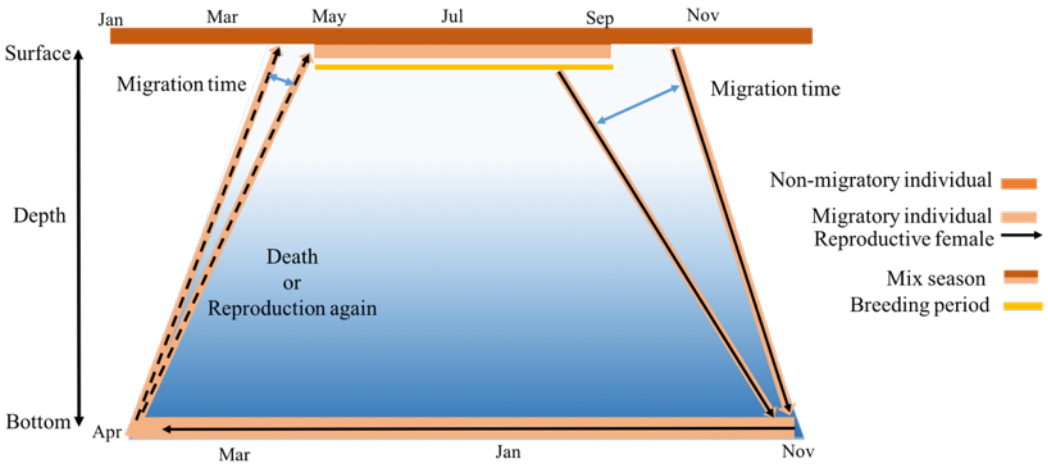

Figure 6.1. Life history of migratory and non-migratory individuals of *Palaemon paucidens* in Lake Biwa.

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