Doctoral Thesis

Exploring migration hotspots, timing, and health conditions of the vulnerable *Opsariichthys uncirostris uncirostris* (Hasu fish) during its reproductive season in Lake Biwa tributaries with three novel techniques: eDNA, stable isotope analysis and micro-CT

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Abstract

Reproductive migration is vital for the continuation of a species. Unfortunately, the reproductive migration of potamodromous fish, such as *Opsariichthys uncirostris uncirostris* (Hasu fish), has not be extensively studied due to difficulties in collecting information. Hasu fish, an endemic piscivorous species to Lake Biwa, plays a key role in maintaining ecosystem function in the Lake Biwa ecosystem. However, the species has been on a continued decline in population size for the past 70 years and is considered extinct in some of its native habitats. To better understand the reproductive migration needs of Hasu fish, this an integrated study consisting of three novel techniques: eDNA, stable isotopes and micro-CT, was conducted to identify migration hotspots, explore the differences in the timing of upstream migration between individuals and examine the health status of the fish during its reproductive migration to Lake Biwa tributaries. Using eDNA copies and visual inspection, Hasu fish was found to mostly migrate (with peak migration between July and August) in rivers on the northwestern side of Lake Biwa. These rivers, e.g., the Ado, Shiotsuo and Chinai rivers, had fast flowing currents, sandy or gravel bottom substrates, all of which are key for the reproductive success of Hasu fish. Unlike previous studies, this study also found that the number of eDNA copies in some rivers increased slightly in September, therefore suggesting late migration by some individuals. Using biometric measurements, migrating Hasu were found to be at least 37% larger in standard length than had been reported in the 1960s by Tanaka. For the first time ever, this study documented feeding behavior of Hasu fish during its reproductive migration, especially on Ayu fish. However, using $\delta^{15}N$ and $\delta^{13}C$ isotopes and the catch data, revealed differences in the onset of feeding (and possibly upstream migration) between individuals from Lake Biwa to the rivers, with males likely arriving and feeding before females, probably to prepare for spawning. All migrating Hasu individuals were health ($K > 1$), however, bone density analysis using micro-CT revealed differences in bone density between sexes, with females having higher bone density than males. Moreover, the bone density decreased slightly in both sexes with increasing standard length. These findings highlight the role of age and sex on the health status of fish and suggests resource utilization strategies by Hasu fish. In addition, the bone density changes provides strong evidence that Hasu fish can adapt to stressful events during reproductive migration. For the effective conservation of Hasu fish and the Lake Biwa ecosystem, the findings from this integrated research should be considered.

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1 Introduction

1.1 Chapter summary

In this chapter, I review the history of migration studies and its ecological significance. I also review the challenges faced by conventional methods and introduce innovative approaches to studying migration. I also review the role of the potamodromous Hasu fish in the Lake Biwa ecosystem, some challenges currently facing the fish and why it is important to study reproductive migration of the fish. Finally, I outline the objectives of this doctoral thesis.

1.2 History of migration studies

Migration is an important aspect of many fish species, and for generations, mankind has been able study migratory fish. To produce a comprehensive study on migratory animals, it is important that one understands the meaning of the term “migration” because; the definition used influences research tools. The definition of migration varies greatly between studies. For instance, Fryxell (1991) describes migration as a seasonal movement of animals tracking changing resources; Hanson and Hylander (2009) describe migration as a periodic diel movement of organisms between two environments and Holland et al. (2006) describe migration as one-way movements of single generations that may, over multiple generations, lead to a return of an entire population to its point of origin. Borrowing from these definitions and for purposes of this research, migration will be defined as the seasonal movement of animals between two environments in which the migrating animal returns to its point of origin (with emphasis on fish as migratory animals). The significance of this definition is its emphasis on both the temporal (i.e., season) and spatial (i.e., two environments) aspects of migration (Thurrow 2016).

The study of animal migration spans centuries, reflecting humanity's enduring fascination with the natural world. While ancient civilizations observed the seasonal movements of birds and mammals, it was not until the 19th century that systematic

investigations into animal migration became scientifically recognized (Hoare 2009, Irving 2012). Pioneering ornithologists, such as John James Audubon and William Henry Hudson, made detailed reports on their observations of bird migrations, laying the groundwork for thes field (Barrow 2000). The arrival of technological innovations, particularly bird banding techniques in the early 20th century by scientists like Hans Christian Mortensen, provided researchers with unprecedented tools to track individual animals (Preuss 2001). This marked a new era in migration studies, enabling scientists to document the migration of various species. In the mid-20th century, the rise of radar technology, allowed researchers expand their focus beyond birds to study migration patterns of insects and bats (Irving 2012, Hüppop et al. 2019). This facilitated the establishment of research centers and foster international collaborations, such as the International Biological Programme, which aimed to comprehensively understand the complexities of animal migration (Kwa 1987). As ecological awareness grew, conservationists recognized the importance of migratory routes and habitats, leading to the formulation of policies and initiatives to protect critical areas for migratory species (Kwa 1987, Amezaga 2002). Today, with the further advancement in technologies, and data analysis, scientists continue to push the boundaries of migration studies, unveiling the critical role migration plays in the survival and ecological balance of diverse species around the world (Amezaga, 2002).

1.3 Ecological significance of migration

Animal migration has immense ecological significance, playing an important role in shaping the structure and functioning of ecosystems across the planet (Amezaga, 2002). Perhaps most notably, migratory species play a role in the transfer of nutrients, energy, and genetic diversity between ecosystems (Bauer & Hoye 2014). As animals move between ecosystems, they transport vital nutrients, such as nitrogen and carbon, linking different ecosystems and influencing nutrient cycling dynamics (Knops et al. 2002). In aquatic ecosystems, anadromous fish, like salmon, carry marine nutrients upstream to freshwater bodies,

where they may deposited through excretion, spawning (Bauer & Hoye 2014). Similarly, herbivorous migrations on land, such as the great Serengeti wildebeest migration, disperse nutrients through their waste, influencing soil fertility and plant composition in different ecosystems (Knops et al. 2002, Subalusky et al. 2017). Animal migrations also serve as key drivers of population dynamics and trophic interactions (Bauer & Hoye 2014). For instance, the arrival of a prey species, can elicit feeding frenzies in resident predators, thereby affecting the population dynamics and distribution of resources (James 1988, Knops et al. 2002). Moreover, migrations can also act as a regulatory mechanism for prey species, preventing overgrazing in certain areas while promoting biodiversity in others (Subalusky et al. 2017). The interconnectedness of these migratory processes promotes resilience in ecosystems, making them more adaptable to environmental changes (Bauer & Hoye 2014). As such, the conservation and understanding of animal migrations are important for maintaining the health and stability of ecosystems.

1.4 Reproductive migration in aquatic ecosystems

Reproductive migration is a specialized and critical branch within the broader field of animal migration studies. Reproductive migration, wherein species undertake extensive journeys specifically for breeding purposes, offers unique insights into the evolutionary pressures, ecological adaptations, and life history strategies that shape the reproductive success of organisms (Bauer & Hoye 2014). From reproductive migration studies, researchers gain a deeper understanding of the selective forces that have driven the evolution of such behaviors (Leggett 1977, Turbek et al. 2018). The study of reproductive migration is particularly crucial in elucidating how environmental cues, ranging from seasonal changes to specific geographical features, influence the timing and success of reproduction, providing a perspective on the interplay between organisms and their habitats (Turbek et al. 2018). Moreover, reproductive migration research contributes significantly to the conservation and management of species with unique life history traits (Turbek et al. 2018). Many organisms exhibit highly specialized

reproductive migration behaviors, often undertaking dangerous journeys to reach specific breeding grounds (Bauer & Hoye 2014, Subalusky et al. 2017, Turbek et al. 2018). Understanding the factors influencing the success of reproductive migrations enables conservationists to identify and protect critical habitats and corridors essential for breeding events (Kwa 1987, Hoye 2014, Subalusky et al. 2017, Turbek et al. 2018). Given the increasing anthropogenic pressures on ecosystems, from habitat degradation to climate change, studying reproductive migration is a necessary tool for designing effective conservation strategies that safeguard the continuation of species, ultimately contributing to the ecological balance of ecosystems (Kita et al. 2006).

Migration in aquatic ecosystems generally falls into two broad categories: migration between seawater and freshwater systems (diadromy), and migration entirely within freshwater systems (potamodromy) (Thurrow 2016). In the case of fish, rare and endangered potamodromous fishes have not been sufficiently studied due difficulties in collecting information (Lucas & Baras 2008). In particular, the reproductive migration of potamodromous fish have not been sufficiently studied despite their ecological significance (Lucas & Baras 2008, Benitez et al. 2015, Thurrow 2016). The limited research on potamodromous species, including but not limited to various freshwater fish, has left gaps in our understanding of the ecological dynamics associated with their reproductive migrations (Lucas & Baras 2008, Benitez et al. 2015, Thurrow 2016). The scarcity of data on the ecological consequences of potamodromy hinders our ability to formulate comprehensive conservation strategies for these species and the freshwater ecosystems they inhabit (Benitez et al. 2015). Addressing the research gap in potamodromous species reproductive migration is critical for the improving the management of aquatic ecosystems (Thurrow 2016). These species play integral roles in nutrient cycling, energy transfer, and the overall functioning of freshwater ecosystems (Bauer & Hoye 2014, Wheeler et al. 2015, Thurrow 2016). A thorough investigation into the reproductive migrations of potamodromous species would provide valuable insights into their

life history strategies, habitat requirements, and potential vulnerabilities (Lucas & Baras 2008, Turbek et al. 2018). By taking advantage of technologies and interdisciplinary research, it may help unveil the intricacies of the reproductive migration of potamodromous species, and thereby contribute to a more comprehensive understanding of the ecological dynamics within freshwater systems.

1.5 Rationale for selecting research tools in migration research

The rationale for selecting research tools in migration research is rooted in the pursuit for precision, efficiency, as well as ethical considerations (Lucas & Baras 2008, Barglowski 2018). Researchers must carefully choose tools that align with the specific objectives of their studies, considering the scale and scope of the migratory phenomenon under investigation (Vargas-Silva 2012). The selection process typically involves a balance between accuracy and minimizing interference with the natural behaviors of the studied species (Barglowski 2018). For instance, when studying large-scale migrations, telemetry can be considered as a suitable tool, due to its ability to capture expansive movement patterns (Griffin et al. 2020). On the other hand, in scenarios where detailed individual behaviors are required, advanced tracking technologies like GPS devices may be required to provide high-resolution data (Fiedler 2009). Moreover, the ethical implications of the chosen tools must carefully weighed, ensuring minimal disruption to the migratory species and their habitats (Barglowski 2018, Turbek et al. 2018). To ensure a balance between accuracy and interference, two or more methods can be used together as complementary methods (Tverin et al. 2009). For example, in sea turtles, GPS may be used to provide accurate spatial information of the turtles, while telemetry may be used to enhance the understanding of their behavior in relation to the marine environment (Godley et al. 2008). Such combined data can expedite conservation efforts by identifying key areas for protection and helping to mitigate threats to sea turtle populations. Thus, the rationale for selecting tools in any migration research should extend beyond the immediate research goals. It should aim to support conservation efforts by providing ethical and scientific data to enhance

the comprehension of migration dynamics.

1.6 Limitations of conventional methods in migration research

Conventional methods, while still informative, are associated with several limitations that can limit our understanding of the complex dynamics involved in species migrations (Phillips & Eldridge 2006, Hobson & Norris 2008, Klassen et al. 2010, Maruyama et al. 2018). A significant challenge lies in the inability of these methods to capture fine-scale details of individual movements and behaviors (Morelle et al. 2017). Conventional methods, such as visual observations and mark-recapture techniques, often provide limited spatial and temporal resolution (Phillips & Eldridge 2006, Maruyama et al. 2018). For instance, attempting to track the precise foraging patterns or daily routines of a highly elusive or endangered species can be challenging with these methods, leading to gaps in our knowledge regarding the micro-scale interactions between individuals and their environment. In addition, the reliance on direct observation or physical tagging often introduces bias and ethical concerns (Maruyama et al. 2018, Hüppop et al. 2019). In cases where researchers have used physical tags or collars, the added weight has been documented to influence the natural behaviors of the studied species (Jewell 2013). Such interference may alter migration patterns, feeding habits, or reproductive success, potentially skewing the results of a study. Furthermore, direct observations may be impractical for certain species or in remote or challenging environments, limiting the scope and accuracy of the gathered data (Maruyama et al. 2018). For example, tracking marine species like whales over vast ocean expanses can be logistically demanding and cost-prohibitive using conventional methods, leading to incomplete insights into their migratory behavior (Lewis et al. 2017). The temporal limitations of conventional methods also pose challenges in capturing the complexity of migration (Phillips & Eldridge 2006, Heady & Moore 2013). Many species exhibit dynamic responses to environmental changes over time, and seasonal nuances in migration patterns may be missed with sporadic observations (Morelle et al. 2017). In such cases, long-term studies become essential for understanding the variations in migration routes,

timing, and behaviors (Lewis et al. 2017). However, there are many times when critical conservation decisions need to be made, leaving researchers devoid of the luxury of long-term research (Heady & Moore 2013). In this regard, newer technologies, such as GPS tracking, environmental DNA, stable isotope analysis and micro-computed tomography, allow for continuous, high-resolution data collection, overcoming these temporal limitations and providing a more comprehensive understanding of migration dynamics (Godley et al. 2008, Heady & Moore 2013, Maruyama et al. 2018). The next section discusses some of these newer technologies with a special focus on reproductive migration in aquatic systems.

1.7 Innovative and novel approaches to studying reproductive migration of vulnerable fish: eDNA, stable isotopes and micro-CT

In recent years, technological innovations have revolutionized the field of migration research, providing scientists with unprecedented tools to explore the intricacies of animal movements (Amezaga, 2002). This technology has been instrumental in studying the migration of birds, mammals, and even marine species, offering insights that were previously unattainable through conventional methods (Heady & Moore 2013, Maruyama et al. 2018, Yamanaka et al. 2018). When it comes to studying vulnerable fish, a number of innovative and novel approaches have emerged that, compared to conventional methods, are universal (i.e., not taxon-specific and applicable to different species), inexpensive (i.e., significantly reducing sampling time and labor efforts), and non-invasive, allowing their application in the ecosystem with little or no damage (Phillips & Eldridge 2006, Hobson & Norris 2008, Klassen et al. 2010, Maruyama et al. 2018). Among the many innovative techniques that have been developed for studying vulnerable fish over the years, this study focuses on three approaches: eDNA, stable isotope analysis, and micro-CT, chosen for their efficiency as tools in the study of vulnerable fish.

One technique making significant strides in migration research is environmental DNA (eDNA). This method involves the collection and analysis of genetic material shed by organisms into their environment, such as skin cells, feces, or mucus. By extracting and

analyzing these traces of DNA from water or soil samples, scientists can identify the presence of specific species, including those undertaking migratory journeys (Takahara et al. 2012, Rees et al. 2014, Ficetola 2008). eDNA provides a non-invasive and efficient means of monitoring elusive or endangered species, offering a unique understanding of migration patterns and their ecological implications (Maruyama et al. 2018, Yamanaka et al. 2018). eDNA analysis has also revolutionized how we monitor vulnerable fish populations. By extracting genetic material from the aquatic environment, researchers can detect species presence, assess population dynamics, and identify critical habitats without direct interaction with the fish (Ficetola 2008, Maruyama et al. 2018, Yamanaka et al. 2018). eDNA analysis is largely based on the principle that the environment can preserve molecular imprint of inhabiting species (Takahara et al. 2012, Ficetola et al. 2008). Based on this principle, it is possible to accurately detect organisms without direct observation by using sequencing techniques. eDNA analysis is typically a five-step process: sample collection, filtration, preservation, extraction, and amplification with detection. Thus, period sampling or tracking of eDNA changes in the aquatic ecosystem can help us identify recent migration (Maruyama et al. 2018). Despite its non-invasiveness, the method is not without its challenges. Among others, the challenges include estimating abundance and biomass accurately, the method is also affected by environmental factors like stream flow, pH, temperature, and DNA-degrading microbes. Additionally, eDNA data does not provide information on the previous habitat of the migrating species (Takahara et al. 2012, Rees et al. 2014, Iwai et al. 2018, Harper et al. 2019).

Stable isotopes represent another powerful tool in migration research, giving us a unique perspective on the life history and movements of animals (Hobson & Norris 2008, Heady & Moore 2013). Isotopic analysis involves examining the ratios of stable isotopes in tissues like feathers, fur, or bones, which can provide information about an animal's diet, geographic origin, and migratory history (Hobson & Norris 2008). This technique has been widely applied to migratory birds to understand the linkage between breeding and wintering grounds (Hobson &

Norris 2008, Phillips & Eldridge). By analyzing isotopic signatures, researchers can deduce the geographic origins of migratory individuals, revealing critical information for conservation and management efforts. Stable isotopes have also been used to provide valuable insights into the dietary habits and trophic interactions of vulnerable fish (Sawada et al. 2019). Analyzing the stable isotopic composition of tissues allows researchers to trace the origin of nutrients, understand food web dynamics, and evaluate the ecological niche of these species (Maruyama et al. 2001, Kurasawa et al. 2023). This approach not only contributes to our understanding of the role these fish play within aquatic ecosystems but also sheds light on their adaptability and susceptibility to environmental changes. Similar to eDNA sampling, using stable isotopes in ecological research is a five step process: sample collection, sample preservation, tissue extraction, tissue drying, and isotope analysis. The use of stable isotopes in ecological research gained prominence in the 1990s, notably around the time Maruyama et al. used them to trace gobies in Lake Biwa tributaries. Primarily, stable isotopes have been used to determnine an organism's dietary patterns in the environment (Hobson & Norris 2008). However, Phillips & Eldridge (2006) demonstrated the potential to estimate the timing of diet changes by exploiting tissues with different fractionation rates. According to Phillips & Eldridge (2006), a change in an organism's environment leads to corresponding changes in its tissue isotope composition, allowing us to determine recent migration. However, in order for this to hold, the isotopic signitature of the old and new environmnents must be significantly different (Phillips & Eldridge 2006, Heady & Moore 2013). Additionally, the rates of isotopic turnover, which vary between tissues, must be different for the tissues under investigation. In aquatic ecosystems, mucus tissues generally exhibit faster turnover rates compared to dorsal muscle and fin tissues (Maruyama et al. 2016, Shigeta et al. 2017, Winter et al. 2019). Therefore, using isotope ratios in two tissues with significantly different turnover rates, when combined with knowledge of turnover rates and an understanding of the environment's isotope signatures, one could reasonably deduct a diet change or estimate the time since migration. Despite its promising

applications, the use of stable isotope analysis to study vulnerable organisms has been highly debated. The method is considered invasive as it involves capturing an organism and extracting tissue from it, which may sometimes result in injury and death (Church et al. 2009, Maruyama et al. 2016, Winter et al. 2019).

Micro-computed tomography (micro-CT) represents another innovative tool enhancing our understanding of vulnerable fish ecology. Changes in internal morphology are among the fastest, and usually plastic, responses to environmental stresses, often preceding changes in behavior (Badyaev 2005, Jonsson & Jonsson 2014). Non-destructive imaging technology allows researchers to visualize internal structures in a three-dimensional plane, enabling us to examine skeletal features, assess bone density, and understand physiological adaptations (Broeckhoven et al. 2017, Gutiérrez et al. 2018). In the context of vulnerable fish, micro-CT becomes a valuable tool and provides insights into anatomical traits, potential vulnerabilities to environmental stressors, and the overall health of populations. Just like eDNA and stable isotope analyses, using micro-CT in ecological research can be considered a five-step process: sample collection, sample preservation, preparation for scanning, scanning, and 3D reconstruction. This non-destructive imaging technology is particularly useful in studying the skeletal adaptations of fish undergoing reproductive migrations, shedding light on the biomechanics and energetics associated with migration (Broeckhoven et al. 2017, Vasconcelos-Filho 2019). Therefore, it should be possible to use micro-CT to monitor changes in bone density across a sampling period and shed light on the resource use or mobilization in migrating populations. For instance, researchers can use bone density changes through micro-CT to determine the health of individuals during reproductive migration. According to Wolff's Law on bone remodeling, the structure (and consequently density) of bone tissue in healthy individuals will adjust in response to the mechanical forces and stresses applied to it (Frost 1994). Such information is important for conservation efforts and ensuring that migrating individuals have the necessary resources for reproductive migration.

By combining these three innovative approaches – eDNA analysis, stable isotopes (SI), and micro-CT (μCT) imaging – researchers can achieve a more holistic understanding of the ecological dynamics and conservation needs of vulnerable fish species in their aquatic habitats (Figure 1.1)).

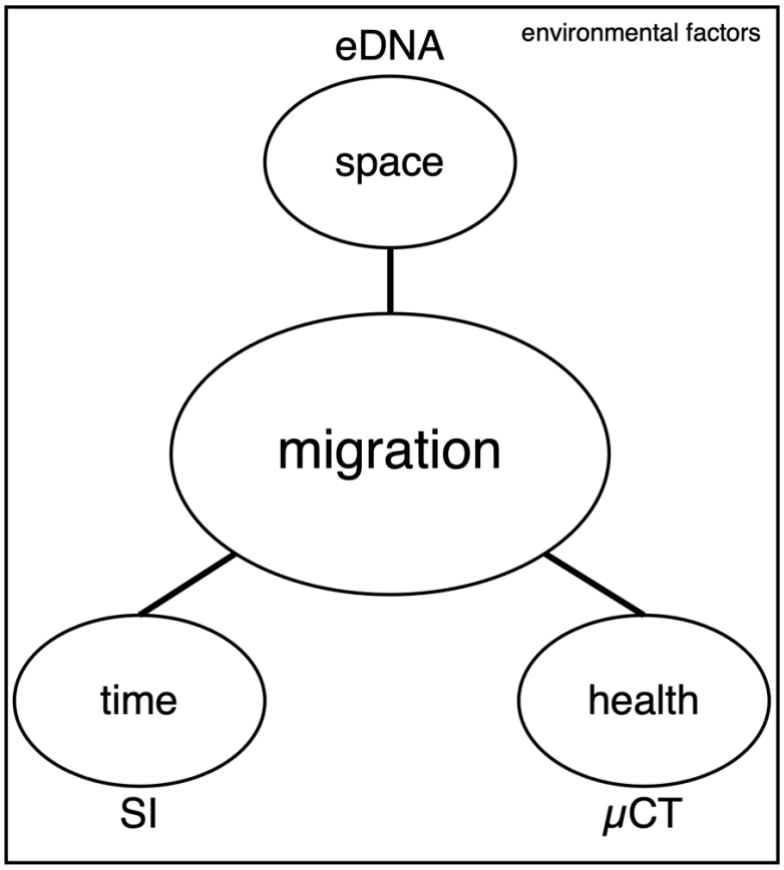

Figure 1.1. A schematic diagram showing how novel techniques integrate to provide a holistic picture of reproductive migration. In this schematic eDNA provides the spatial aspect of migration while stable isotope (SI) analysis provides the temporal aspect and micro-computed tomography (μCT) provides the health aspect. The area around the schematic represents how the various environmental factors affecting this relationship.

1.8 Significance of studying *Opsariichthys uncirostris uncirostris* reproductive migration

Lake Biwa is one of the ancient lakes of the world, 5-6 million years old, and has a modest diversity of organisms (Kawanabe 1996). Unfortunately, the water quality in Lake Biwa deteriorated significantly in the 1960s due to rapid population growth, inadequate wastewater treatment, and agro-chemical abuse, all of which are key drivers of eutrophication in lakes (Kita et al. 2006). Some of the lake's tributaries have also been redirected, while others reconstructed

to accommodate human needs. Reconstruction has also seen the introduction of concrete riverbanks and substrates in some rivers potentially disrupting the ecosystem (Kita et al. 2006, Ministry of Land, Infrastructure, Transport and Tourism, Japan 2016a, 2016b). Despite efforts by the local government to prevent further deterioration of the lake's ecosystem, ecosystem restoration has been slow (Kita et al. 2006). This is a serious concern and poses a threat to the endemic species in the Lake's ecosystem. One such endemic species facing the threat is *Opsariichthys uncirostris uncirostris*.

Opsariichthys uncirostris uncirostris (local name: Hasu) is a potamodromous species whose reproductive migration has not been extensively studied since the 1960s. The last comprehensive study on the species was conducted by Tanaka in 1964. Hasu relies on lake-to-river migration for reproduction, and it is the only piscivorous cyprinid fish in Japan, endemic as a sub-species to Lakes Biwa and Mikata. Hasu fish is reported to spawn in summer from late May to early August in the shores of Lake Biwa and its tributaries, with mostly mature males and females of ages 3 years (average body length: 160 mm) and 2 years (average body length:130 mm), respectively. As an apex predator, Hasu fish is vital for maintaining ecosystem function in its ecosystem. For example, Hasu fish helps control populations of prey species, such as Ayu fish and gobies (Tsunoda et al. 2015). Hasu fish has also been documented to facilitate the cycling and transfer of nutrients within the Lake Biwa ecosystem during its reproductive migration (Kurasawa et al. 2023). As predators consume prey, they may physically store the nutrients, translocate them, or release them back into the environment through excretion. Predators can also elicit antipredator responses in prey, leading to spatial and temporal redistribution of nutrients through changes in prey habitat and foraging behavior shifts (Schmitz et al. 2010).

In many aquatic ecosystems, the role of apex predators in critical ecosystem functions is often overlooked. Apex predators are harvested unsustainably, and their numbers have been greatly reduced (Heithaus et al. 2010, Schmitz et al. 2010, Atwood et al. 2015). Unfortunately,

Hasu fish has also not received much attention compared to fishery species such as Ayu fish, and its population has been steadily declining for the past 70 years (Figure 1.2).

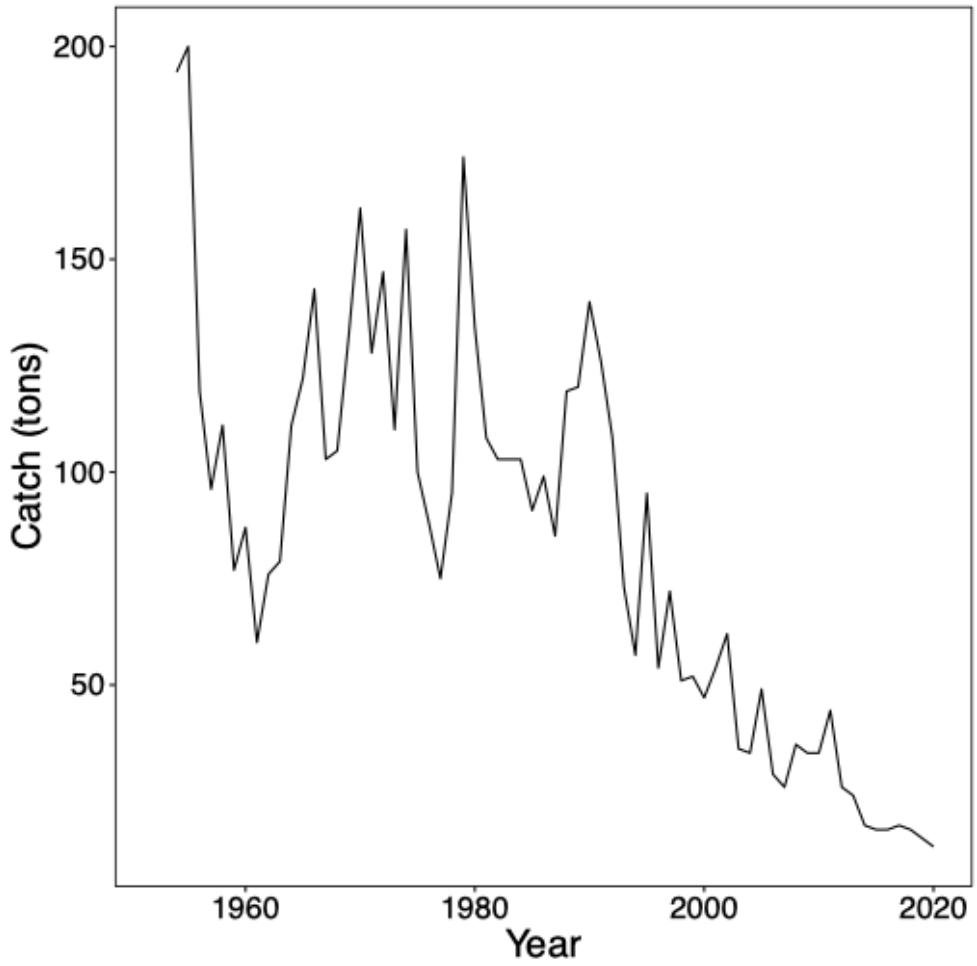

Figure 1.2. The declining trend of Hasu fish catches in Lake Biwa from 1954 to 2020

The species is considered vulnerable in Lake Biwa and extinct in Lake Mikata (Maruyama et al. 2018, Ministry of the Environment Japan 2020). If the population of lower-level consumers, such as Ayu fish in Lake Biwa, is left unchecked, it may lead to a trophic cascade and cause ecosystem dysfunction (Heithaus et al. 2010). A comprehensive study on Hasu fish is thus required to better understand why the species has been in continued decline for the past 70 years. However, given its vulnerable status and highly evasive nature, a combination of novel techniques such as eDNA, stable isotope analysis, and micro-CT becomes a valuable tool for studying Hasu fish with minimal disruption to the species, while maintaining the ecosystem function in Lake Biwa.

1.9 Research justification

To effectively conserve rare and endangered species, such as the vulnerable potamodromous Hasu fish, understanding their reproductive migration is important. Successful reproduction plays a fundamental role in ensuring the species' continued existence. A comprehensive reproductive migration study must carefully address key elements, such as the timing of migration, health of individuals and locations of reproduction. Neglecting these critical elements may lead to the species being adequately protected in certain areas and times but facing threats in others. Regrettably, numerous studies fall short in encompassing all necessary elements of reproductive migration, either focusing narrowly on a single element or providing incomplete accounts due to resource and time constraints. This research sought to address this gap by undertaking a holistic examination of the critical elements (i.e., space, time, and health) in the reproductive migration of the potamodromous Hasu fish. Through the integration of novel techniques, this study provided a comprehensive understanding in a single investigation, contributing valuable insights, not only into the conservation of this species, but many others facing similar challenges.

1.10 Research objectives

The main objective of this research was to the assess the reproductive migration of the vulnerable potamodromous Hasu fish by usin three novel techniques as complementary methods in one study.

The specific objectives were to:

- identify reproductive migration hotspots of Hasu fish in Lake Biwa tributaries using eDNA & visual counts.
- demonstrate differences in timing of upstream migration to Lake Biwa tributaries in Hasu fish and explore food habits using SIA and biometrics.
- explore the health of the migrating Hasu fish population using variations in bone density.

2 Identifying migration hotspots of Hasu fish in Lake Biwa tributaries using environmental DNA and visual counts during its reproductive season

2.1 Chapter summary

I explored migration hotspots of the vulnerable Hasu fish in Lake Biwa tributaries using two complementary methods: environmental DNA (eDNA) and visual counts. The main objective of this study was to identify rivers that are important for Hasu fish reproductive migration. The study encompassed the known range of Hasu fish around Lake Biwa tributaries during its reproductive season. Monthly water sampling and visual inspection was conducted, from May to September, in 32 Class A tributaries—at the river mouth and within the river channel. Hasu eDNA was extracted from water samples and quantified using real-time Polymerase Chain Reaction (PCR). Environmental factors were also assessed on-site, and their effects on eDNA and visual count trends evaluated using linear models and Akaike information criterion (AIC). eDNA was detected in sites where the fish were both observed and not observed. Using a zero-hurdle model revealed positive correlation between eDNA copies and visual counts of migrating Hasu, with pH, a determinant of Hasu fish presence in rivers, having a reducing effect on the relationship. Analysis of Hasu eDNA copies and visual count trends, with environmental factors as explanatory variables, indicates that Hasu fish are likely to be found in rivers that are wide and deep enough to accommodate migrating individuals, have fast-flowing currents, and sandy-gravel substrates during reproductive migration. Such rivers are mostly located on the western side of the northern basin and include the Ado, Chinai and Shiotsuo rivers. These could be considered as Hasu fish migration hotspots and require protecting if the population of Hasu fish in Lake Biwa is to be recovered.

2.2 Introduction

Studies on fish migration have revealed that fish migrate for various reasons, including

establishing new feeding grounds, escaping unfavorable environmental conditions, and spawning—all of which are vital for the continued existence of a species (Fryxell 1991, Holland et al. 2006, Hansson & Hylander 2009, Thurrow 2016). However, despite tremendous advances in migration studies, considerable challenges remain when applying conventional methods to rare and elusive species. For instance, potamodromous fishes, which migrate entirely freshwater systems, have not received sufficient attention due to difficulties in collecting information (Lucas & Baras 2008, Thurrow 2016). These difficulties include challenges like visual counts, which are not easily obtained when the population size is extremely low, and Sr/Ca ratio, which are not applicable to potamodromous fishes.

In recent years, novel techniques, such as environmental DNA (eDNA) analysis, have gained traction in migration studies. eDNA can be extracted from environmental samples, such as sediment and water (Ficetola et al. 2008). The application of eDNA to migration studies is relatively new and is based on the principle that the environment can preserve the molecular imprint of inhabiting species (Ficetola et al. 2008). According to this principle, it is possible to detect organisms without direct observation by using extracted DNA from an environmental sample and conducting species-specific PCR detection or quantification. The results from eDNA analysis have helped to identify recently migrating organisms in freshwater systems and, consequently, migration hotspots, defined in this paper as areas with high eDNA copies and fish counts during the reproductive season. This is particularly applicable when sampling is done in surface water, where eDNA can persist for up to a week even after the migrating organism has left the environment, as opposed to sediments, where eDNA has been detected up to 3 months after the migrants have left the environment (Maruyama et al. 2014, Turner & Everhart 2015). Periodic sampling and monitoring of eDNA copies can also help determine seasonal migration (Laramie et al. 2015, Wacker et al. 2019). Hypothetically, eDNA copies will increase with the arrival of new individuals, remain at a dynamic equilibrium when abundance is sustained, and decrease with the departure of individuals. Additionally, eDNA should be absent with the

absence of the individuals.

While the application of eDNA in freshwater systems appears to be established, the method faces significant obstacles. One such obstacle in current eDNA studies is its reliability in estimating abundance (or biomass) in migrating populations based on eDNA samples (Takahara et al. 2012, Rees et al. 2014, Maruyama et al. 2018, Harper et al. 2019). Accurate abundance (or biomass) estimates are crucial for making decisions regarding the management and conservation of rare and endangered species. The reliability of this method has been a subject of debate in lotic systems. Iwai et al. (2018) observed that stream flow prevented the even distribution of eDNA, making it difficult to obtain eDNA copies that accurately reflected the number of individuals present in the environment. However, Maruyama et al. (2018) found that stream flow did not have a significant effect on eDNA quantification in the environment. Nevertheless, taking into consideration environmental factors such as pH and temperature, as well as incorporating other methods like visual counting, can improve the efficiency of eDNA analysis as a tool for studying migrating rare and endangered species.

Opsariichthys uncirostris (local name: Hasu fish) is a potamodromous species that has not been extensively studied since the 1960s. The last comprehensive study on the species was conducted by Tanaka in 1964. Hasu fish rely on lake-to-river migration for reproduction, and it is the only piscivorous cyprinid fish in Japan, endemic as a sub-species to Lakes Biwa and Mikata (Tabata et al. 2016). Unfortunately, its population has been steadily declining for the past 70 years (Figure 1.2). Consequently, Hasu is considered vulnerable in Lake Biwa and extinct in Lake Mikata (Ministry of the Environment, Japan, 2020). Hasu spawning is reported to occur in summer, from late May to early August in Lake Biwa and its tributaries (Tanaka 1964, Miura 1966). Spawning takes place over sandy-gravel bottoms within inlet streams and the shores of Lake Biwa, primarily involving mature males and females of ages 3 years (average body length: 160 mm) and 2 years (average body length:130 mm), respectively. Using eDNA analysis, Maruyama et al. (2018) deduced that during the reproductive season in the Chinai River, located

on the north-western side of Lake Biwa, the abundance of Hasu fish gradually increases from May to July and decreases in August. Maruyama et al. (2018) also argued that Hasu fish inhabit spawning sites for longer times than previously reported. However, Maruyama et al.'s (2018) study was conducted in only one of 117 Class A tributaries (rivers under the control of the National Government) of Lake Biwa and may not provide a complete picture on the range of Hasu fish migrations in the Lake Biwa ecosystem. A comprehensive study covering a wider range could help identify areas of conservation importance and shed light on why the Hasu fish population has continued to decline for over 70 years.

Therefore, this study aimed at identifying key rivers (migration hotspots) of the vulnerable potamodromous Hasu fish in Lake Biwa tributaries by using visual counts and eDNA analysis as complementary methods within a single study. Water samples were periodically collected for eDNA analysis, and Hasu fish counts (by visual inspection) were concurrently obtained in 32 of the 117 Class A tributaries of Lake Biwa. The study encompassed the range of the species in tributaries on all sides of Lake Biwa. We then used environmental factors to evaluate the trends in Hasu fish counts and eDNA copy numbers within the 32 tributaries, with the aim of uncovering what parameters made the migration hotspots conducive for Hasu fish migration.

2.3 Materials and Methods

2.3.1 Field sampling

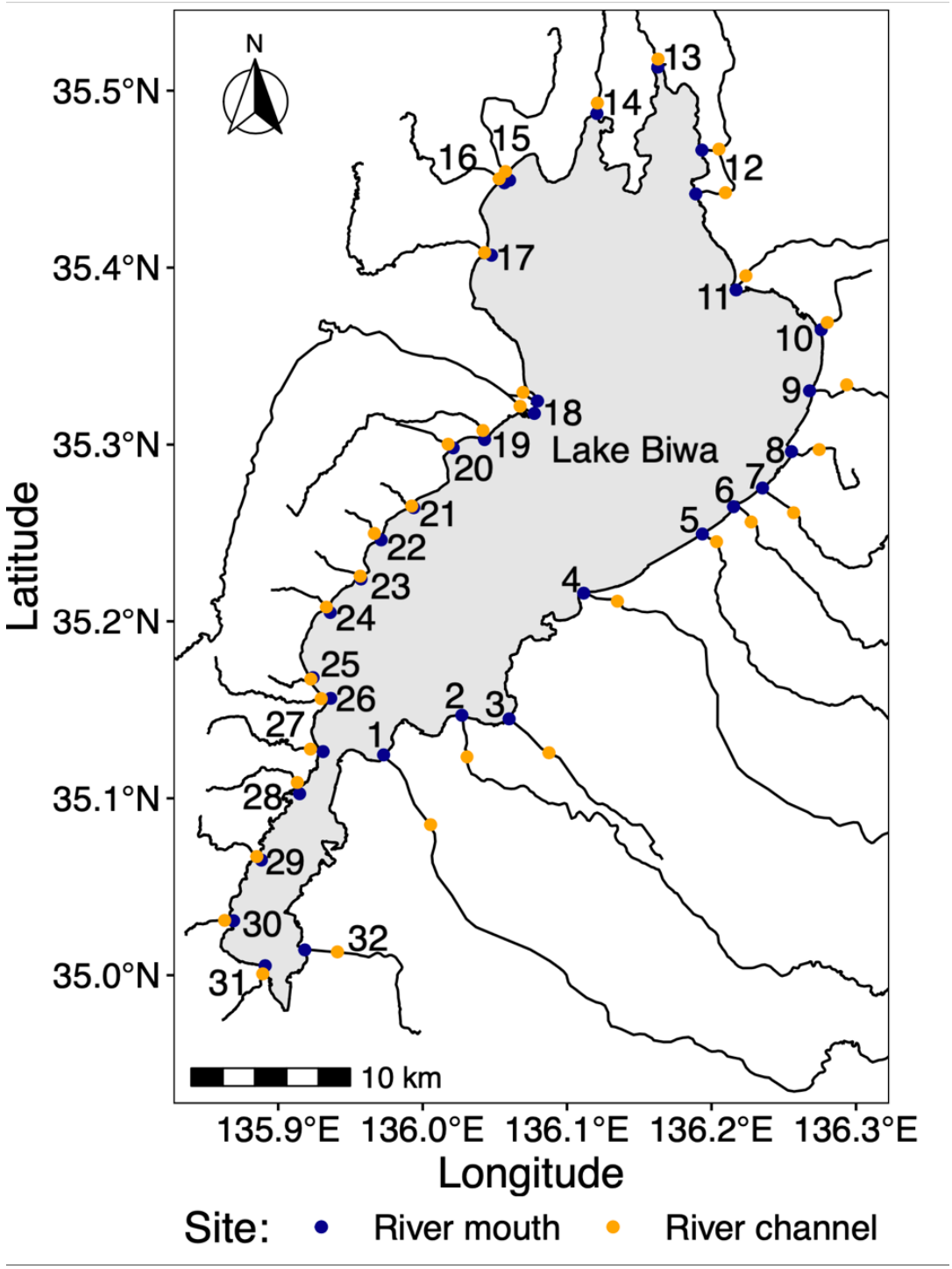

Figure 2.1. Location of sampling sites in Lake Biwa tributaries investigated for Hasu migration in this study. The numbers 1–32 indicate: Yasu (1), Hino (2), Shiratori (3), Echi (4), Uso (5), Inukami (6), Seri (7), Yagura (8), Amano (9), Nagahamashinsen (10), Ane (11), Yogo (12), Shiotsuo (13), Oura (14), Chinai (15), Momose (16), Ishida (17), Ado* (18), Kamo (19), Wadauchi (20), U (21), Taki (22), Hira (23), Otani (24), Kisen (25), Wani (26), Mano (27), Tenjin (28), Omiya (29), Yana (30), Sagami (31), and Kusatsu (32) rivers, respectively. *River has two arms feeding into L. Biwa. Both arms were independently assessed in the study.*

Water samples for eDNA analysis were collected once a month, from early May to early September 2019, in 32 preselected rivers out of 117 Class A rivers feeding into Lake Biwa (latitude: 35.00° N to 35.52° N; longitude: 135.86° E to 136.29° E)—selection of the rivers was done based on previous sightings of the fish as well as to encompass all sides of Lake Biwa. Water samples were collected during the daytime within a uniform time range (e.g., 9 am to 10 am), but the time ranges varied among the different rivers. When sampling was not possible due to bad weather, sampling was rescheduled for the next available date. Water samples were collected at the river mouth and within the river channel close to the river mouth (Figure 2.1). Sampling stations within the river channel were selected based on the presence of a bridge (for easy visual inspection) and absence of backflow. During each sampling, 1 L of water was collected using a polyethylene cup and filtered on site using a glass fiber filter (Whatman GF/F, 0.7 μm nominal pore size, GE Healthcare, Chicago, US) and a polypropylene filter holder (FH-PP47, ASONE, Osaka, Japan) immediately after sampling to reduce eDNA decay—none of the equipment was reused during the course of a sampling day to avoid inter-sample contamination; the polyethylene cups were one-time-use and appropriately discarded while the filter holders were bleached with 10% bleach solution before reuse on another sampling day (Yamanaka et al. 2018). Each water sample was handled with a new pair of disposable gloves. During filtration, another new pair of disposable gloves was used to avoid sample contamination. Remaining water in the glass fiber filter was then replaced with 99.9% ethanol by filtering approximately 2 mL of ethanol (enough to cover the entire surface of the GF/F on the filter holder) to preserve eDNA (Minamoto et al. 2015), folded in half using forceps, and wrapped in aluminum foil to avoid post sampling contamination and light. During each sampling day, no decontamination by bleaching was done in the field, instead, a new pair of forceps was used for each sample. The wrapped filters were put in separate plastic bags to avoid inter-sample contamination and kept below −20°C using icepacks in a cooler box during transportation to the laboratory. At the end of each sampling day, 1 L of distilled water was filtered as a negative

control and treated in the same manner as the samples. At the laboratory, the filters were kept below −20°C in a freezer until eDNA extraction.

2.3.2 Measurement of environmental factors and visual counts

Water temperature (to the nearest 0.01 °C), pH, and electrical conductivity (to the nearest 0.01 μS/cm) were measured and collected on site during each sampling using the Hanna HI98130 combo meter (Hanna Instruments Inc., USA); turbidity (NTU) using the Eutech TN-100 turbidity meter (Thermo Scientific, USA); type of substrate (i.e., clay: <0.002 mm, sand: 0.002-2 mm or gravel : >2mm; Xu 2004; a binary system was used to indicate presence or absence of sand (S), gravel (G), and clay (C). In this case, if a substrate was sandy-gravel, it was denoted as S:1, G:1, C:0) and river velocity (to the nearest 0.01 cm/s) using the CR-11 current meter (Cosmo Riken Ltd., Osaka, Japan, Lower limit of detection: 4 cm/s); and depth (to the nearest 0.1 m), width (to the nearest 0.1 m), and coordinates (latitude and longitude) using the Huawei P20 lite (Huawei Technologies, Co., Ltd., Shenzhen, China) with the Google Maps application (Google Inc., Cal., USA). Except for the type of substrate (which used the binary system) and coordinates, all environmental factors were collected in triplicates and the averages were used for subsequent analyses. These were selected based on their effect on the amount of eDNA in water bodies and Hasu fish spawning (Miura 1966, Barnes et al. 2014, Maruyama et al. 2018). Visual inspection was only done within the river channel. It was not done at the river mouth due to difficulties in collecting information (e.g., absence of bridges, steep banks, and dense vegetation). Visual inspection was done by counting the number of Hasu individuals within a 40 m stretch of each eDNA sampling station—Hasu individuals migrating upstream are large enough (14–25 cm standard length) to be distinguished from other fish from either the bridge or riverbanks. Individual density per unit area was not assessed in this study. Unlike density models, models based on individual counts can accommodate a wide range of predictor variables and are better suited at handling zero-inflated data as well as overdispersion (Dalrymple et al. 2003). Overdispersion and zero-inflation are among the challenges faced

when statistically analyzing eDNA copies versus individual count data (Maruyama et al. 2018).

2.3.3 eDNA extraction and quantification

Extraction, amplification and quantification of eDNA in the water samples was done as outlined by Yamanaka et al. (2018) and Maruyama et al. (2018) with slight modifications. Thus, extraction of eDNA was done using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Amplification and quantification of eDNA was performed using the real-time PCR system (Applied Biosystems® StepOnePlus™, Thermo Fisher Scientific, Waltham, USA).

Prior to extraction, a reagent mix was prepared by mixing 200 µL Milli-Q-water, 100 µL Buffer AL, and 10 µL proteinase K per sample extraction in a centrifuge tube. Water filters were folded into cylindrical form using forceps and placed in the upper part of spin columns (EconoSpin GDI-EP-31201-250, Funakoshi, Japan), which had their silica gel membranes removed prior to the input. The columns were then centrifuged at 6,000 × *g* for 1 min to remove excess moisture from the filters.

Extraction was done by dispensing 310 µL of the reagent mix onto the filter in each spin column and the columns incubated at 56°C for 30 min. After incubation, the columns were centrifuged at 6,000 × *g* for 1 min to collect eDNA. The eluted filtrate was then transferred to a new collection tube. Residual eDNA on each filter was collected by adding 200 µL TE (Tris-EDTA) buffer to the filter, allowing it to stand for 1 min at room temperature (20–25°C), and centrifuging again in a new collection tube at 6,000 × *g* for 1 min. The upper part of the column containing the filter was thereafter removed and discarded, whereas the filtrate was returned to the column holding the first filtrate by pipetting. Then, 100 µL of Buffer AL and 600 µL of ethanol were added to the pooled filtrate and mixed gently by pipetting. eDNA in each mixture was concentrated using the DNeasy Blood & Tissue Kit, according to the manufacturer's instructions. The whole amount of each mixture was transferred to a column provided in the DNeasy Blood & Tissue Kit. The column was then centrifuged at 6,000 × *g* for 1 min and the filtrate discarded (eDNA fragments are trapped on the silica gel membrane of the column). This

was done twice due to the large volume of the mixture and to ensure that all eDNA was collected. The membrane in the column was washed twice with 500 µL of Buffer AW1 and Buffer AW2 while centrifuging and discarding the filtrate following each wash. Centrifuging was done first at 6,000 × *g* for 1 min and then 20,600 × *g* (max) for 2 min after washing with Buffer AW1 and Buffer AW2, respectively. eDNA was finally eluted from the columns by using 200 µL of Buffer AE and centrifuging at 6,000 × *g* for 1 min. eDNA was stored in Lobind tubes at −20°C until qPCR analysis.

eDNA quantification was performed using the Real-Time TaqMan® quantitative PCR with the StepOne-Plus real-time PCR system (Applied Biosystems® StepOne-Plus™, Thermo Fisher Scientific, Waltham, USA). Amplification and quantification of the mitochondrial D-loop gene 129-bp fragments were done using primers and a TaqMan probe designed by Yamanaka et al. (2018): Oun_Dlp_Forward(5′-CATTTCCTTGCCAGGCTTAATAATA-3′), Oun_Dlp_Reverse(5′-GCAAAAGGGGGCATATATATAAGAGA-3′) and Oun_Dlp_Probe(5′-FAM-**.C.**ATAT**.G.**TTTAT**.C.**T**.C.**AT**.G.**T**.G..C.**ATAA**.C.**-TAMRA-3′), respectively. The **.C.** and **.G.** (in bold) in the probe indicate locked nucleic acids that increase melting temperature. Specificity of the primer-probe set has been previously confirmed by Yamanaka et al. (2018) through PCR using tissue samples from three fish species, namely *Opsariichthys platypus*, *Nipponocypris temminckii,* and *N. seiboldoii*, most closely related to Hasu that occupy the same region. Each TaqMan® reaction contained 900 nM of each primer, 125 nM TaqMan® probe in the PCR master mix (TaqMan® Environmental Master Mix 2.0, Thermo Fisher Scientific), 0.075 µL AmpErase® Uracil N-Glycosylase (Thermo Fisher Scientific), and 2 µL of the DNA template. Total volume of each reaction mixture was 15 µL. The PCR conditions were as follows: 2 min at 50°C, 10 min at 95°C followed by 55 cycles of 15 s at 95°C, and 60 s at 60°C. qPCR was performed in triplicate for each eDNA sample, and the average of each triplicate was treated as the final number of eDNA copies in the sample. The quantification of the number of Hasu D-loop genes in each 2 µL eDNA template was performed using a standard curve. A

dilution series of standards containing 30, 300, 3,000, 30,000, and 300,000 copies of the target sequences was used in triplicate for each qPCR assay. The standards, in which the target sequence was cloned using pEX-K4J1 vector, were provided by a commercial service (Standard Genes, Eurofins Genomics K.K., Tokyo, Japan). In this study, "undetermined" results from the quantification were treated as "0". Therefore, the final eDNA quantity in each sample was determined as an average of its 2 μL eDNA template replicates. Following Maruyama et al. (2018), no arbitrary limits of detection and quantification were set—all positive quantification data were included in the statistical analyses.

2.3.4 Data analysis

All data analyses were conducted in R ver. 4.0.3 software. Plots showing the distribution of Hasu fish eDNA copy numbers and visual counts across the reproductive season were created for each of the 32 tributaries of Lake Biwa using the "ggplot2" and "ggpubr" packages in R.

The relationship between eDNA copy numbers and fish counts (by visual inspection) at the river channel sites was assessed using the 'countreg::hurdle()' function in R. The function produces a count model and a zero-hurdle model which are suitable for zero-inflated data. A quick inspection of the data revealed that 70.6% of the visual count data had zeros (120 zero data points out of 170 total data points). This indicated zero-inflation and justified the use of a zero-hurdle model. Unlike standard count models, hurdle models are based on Bernoulli probability. In hurdle models, there is only a binary outcome of a count variate, i.e., either a zero or nonzero result. If the result is nonzero, then the hurdle is crossed, and the conditional distribution of the nonzero data is regulated by a truncated-at-zero count data model (Dalrymple et al. 2003). Different combinations of $\log_{10}$ transformed eDNA copy numbers and the environmental factors (i.e., pH, electrical conductivity, turbidity, type of substrate, river velocity, depth, temperature, and width) were intuitively added to the model. During pre-analysis, a strong correlation was observed between temperature and pH at the river channel sites (correlation coefficient, r = 0.87). The selection between the temperature and pH models

favored the pH model due to its lower AIC score. However, it is worth noting that temperature can affect the distribution of Hasu fish and its eDNA. In fact, the effect of temperature on eDNA degradation, distribution, and quantity has been documented in several studies, including those by Jo et al. (2019), Bedwell & Goldberg (2020), and Kasai et al. (2020). The impact of varying pH levels on eDNA copy numbers was visually represented by modifying the pH values to correspond to the 25-th, 50-th, and 75-th percentiles from the study in the generated model.

The effect of environmental factors on both eDNA copy number and visual count was individually assessed for river channel sites and river mouth sites (no visual counts were obtained at the river mouth in this study). Except for the correlation temperature and pH at the river channel sites (correlation coefficient, $r = 0.87$), no other strong correlations were observed among the explanatory variables. The correlation coefficients ranged from 0.51 (for depth and width in the river channel) to -0.43 (for presence of sand and presence of clay at the river mouth). Therefore, the explanatory variables were treated as independent from each other. The zero-hurdle model was not used to assess this relationship due to the large number of integer values in eDNA data. Additionally, eDNA copy numbers were estimated from C_t values, indicating that they are not true count values. We acknowledge that using mixing models, such as the generalized linear mixed model (GLMM), would have revealed more effects of environmental factors in the models due to the random effect from rivers. However, mixing models could not be applied in the present study due to an insufficient number of datapoints (34 rivers × 2 sampling sites × 5 months × average of 3 replicates for each measurement = 340 records). This study is the first large-scale study on Hasu fish reproductive migration; as a result, data from all the rivers were pooled to effectively assess the effects of environmental factors on the models.

The analysis was therefore done in two steps. First, the effects of environmental factors on the presence of Hasu fish and its eDNA were assessed using generalized linear models (GLMs) with a binomial logit function. This was implemented by the 'base::glm()' function in R. Variable selection for the final model was then achieved through forward and reverse

stepAIC and by using the 'base::stepAIC()' function in R. In the second part of the analysis, linear models (LMs) built with the 'base::lm()' function, were used to assess the relationship between positive eDNA copy numbers and visual counts as response variables and environmental factors as explanatory variables. Variable selection for the final linear models was also achieved by forward and reverse stepAIC. In all cases, the model with the least AIC score was selected. The effect of depth on eDNA copy numbers at the river mouth was not assessed due to difficulties in collecting information on depth.

2.4 Results

2.4.1 Distribution of Hasu fish and its eDNA in Lake Biwa tributaries across the reproductive season

Hasu eDNA was detected in all 32 rivers at some point during the reproductive season (Figure 2.2). Except for a few mismatches (eDNA not obtained despite visual observation; $n = 5$ out of 50 datasets, derived from an eDNA sample and visual presence at each sampling in the entire study), Hasu eDNA was detected in sites where the fish was visually observed during each sampling. Importantly, there was no amplification of Hasu eDNA in any negative controls prepared in the field as well as those used in qPCR analysis—implying that the effect of inter-sample contamination was negligible.

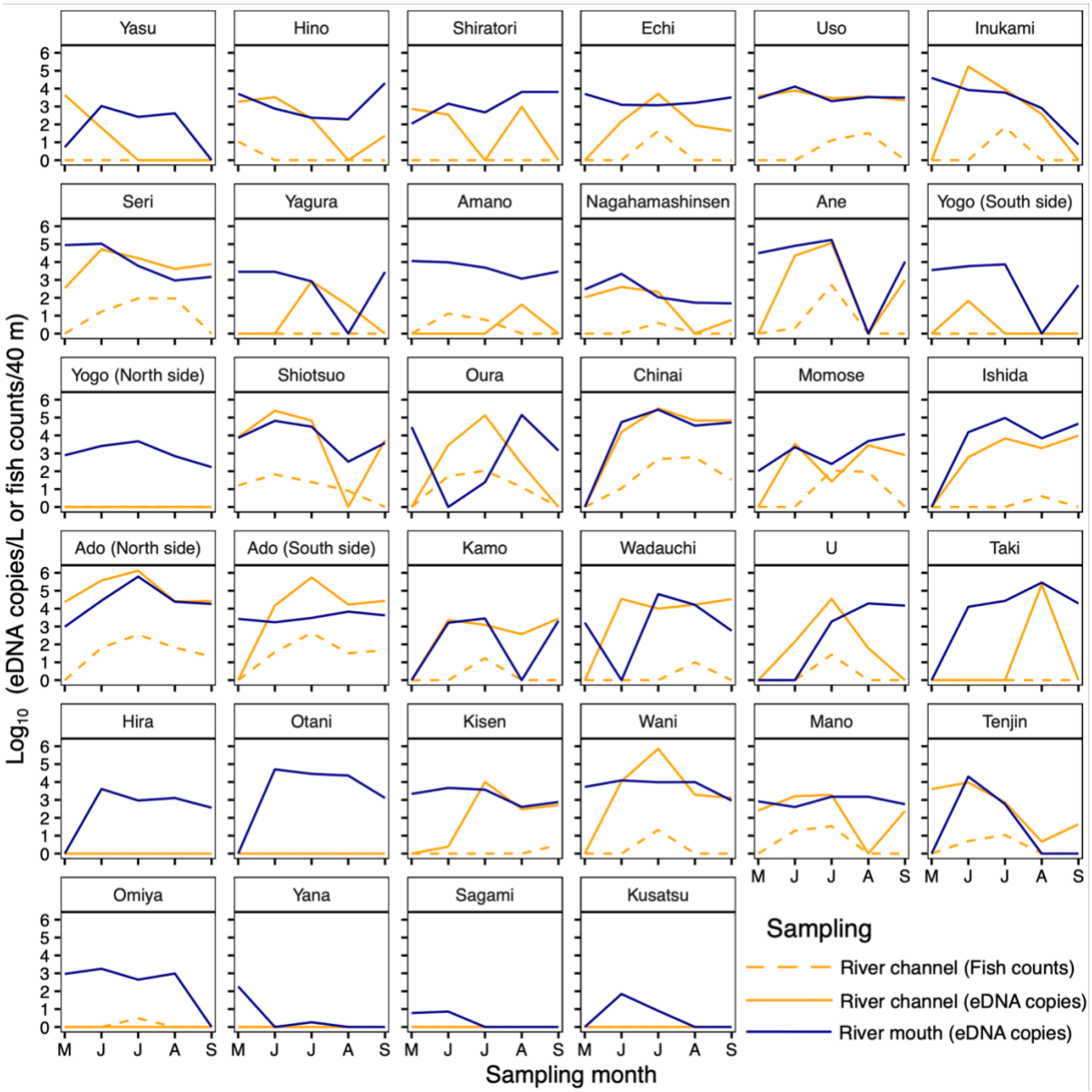

Figure 2.2. Changes in Hasu abundance in Lake Biwa tributaries from May to September revealed by eDNA analysis (log_{10}, copies/L) at the river mouth sites and within the river channel sites and fish counts within the river channel sites (log_{10}, /40 m stretch). The solid blue, solid orange and orange dashed lines indicate: eDNA copies at the river mouth, eDNA copies within the river channel and fish counts within the river channel sites, respectively.

The R^2 values for the qPCR tests ranged from 0.95–0.99 (qPCR efficiency: 64.69%–88.68%). The most individual counts (n = 605 out of 3756 total fish counts in the entire study), in one sampling of Hasu fish at a sampling site, were obtained within the month of August in the Chinai River located on the north-western side of Lake Biwa (Figure 2.2). The highest number of eDNA copies (1.34×10^6 copies/L) were also obtained on the north-western side of Lake Biwa in the northern arm of Ado River within the month of July (Figure 2.2). In some

months and at certain sites, especially on the south-western side of Lake Biwa (i.e., Hira, Omiya, and Taki rivers), no eDNA analysis or visual inspection was conducted due to river drying.

2.4.2 Relationship between Hasu eDNA copies and visual counts

Table 2.1. Coefficients (estimates ± standard errors) for the zero-hurdle model, determined after AIC selection, to estimate individual numbers from eDNA copies and environmental factors. Only datasets from river channel sites were used since no visual inspections were conducted at the river mouth.

	Count model[1]	Hurdle model[2]
(Intercept)	$1.45 \times 10^{0} \pm 4.24 \times 10^{-1***}$	$-2.83 \times 10^{0} \pm 4.28 \times 10^{-1***}$
eDNA (log_{10}, copies/L)	$4.17 \times 10^{0} \pm 1.50 \times 10^{0***}$	$4.44 \times 10^{0} \pm 1.48 \times 10^{0**}$
eDNA (log_{10}, copies/L) : pH	$-4.71 \times 10^{-1} \pm 1.50 \times 10^{-1**}$	$-4.72 \times 10^{-1} \pm 1.91 \times 10^{-1*}$
Log (theta)[3]	$-5.15 \times 10^{-1} \pm 2.85 \times 10^{-1}$	

[1]*Count model assumed truncated negative binomial distribution and used log link function.*
[2]*Hurdle model assumed binomial distribution and used a logit link function.*
[3]*Theta is a measure of overdispersion with respect to the Poisson distribution. Theta N_0: Theta = 1, i.e., there is no excess zeros in the data.*
*Significance levels by Wald tests (***p < 0.001, **p < 0.01, *p < 0.05)*

The zero-hurdle model was used to assess the relationship between visual counts and eDNA copies for only the river channel sites (Table 2.1). No visual count data was obtained at the river mouth due to difficulties in collecting information. The model revealed that estimating the number of Hasu individuals from eDNA copy numbers was positively influenced by the amount of log_{10} transformed eDNA copies ($p < 0.05$). However, when environmental factors were included in the model, the interaction term between log_{10} transformed eDNA copies and pH (pH range: 6.86 – 9.50) had a significant reducing effect on the estimated number of Hasu individuals derived from eDNA copies ($p < 0.05$; Figure 2.3).

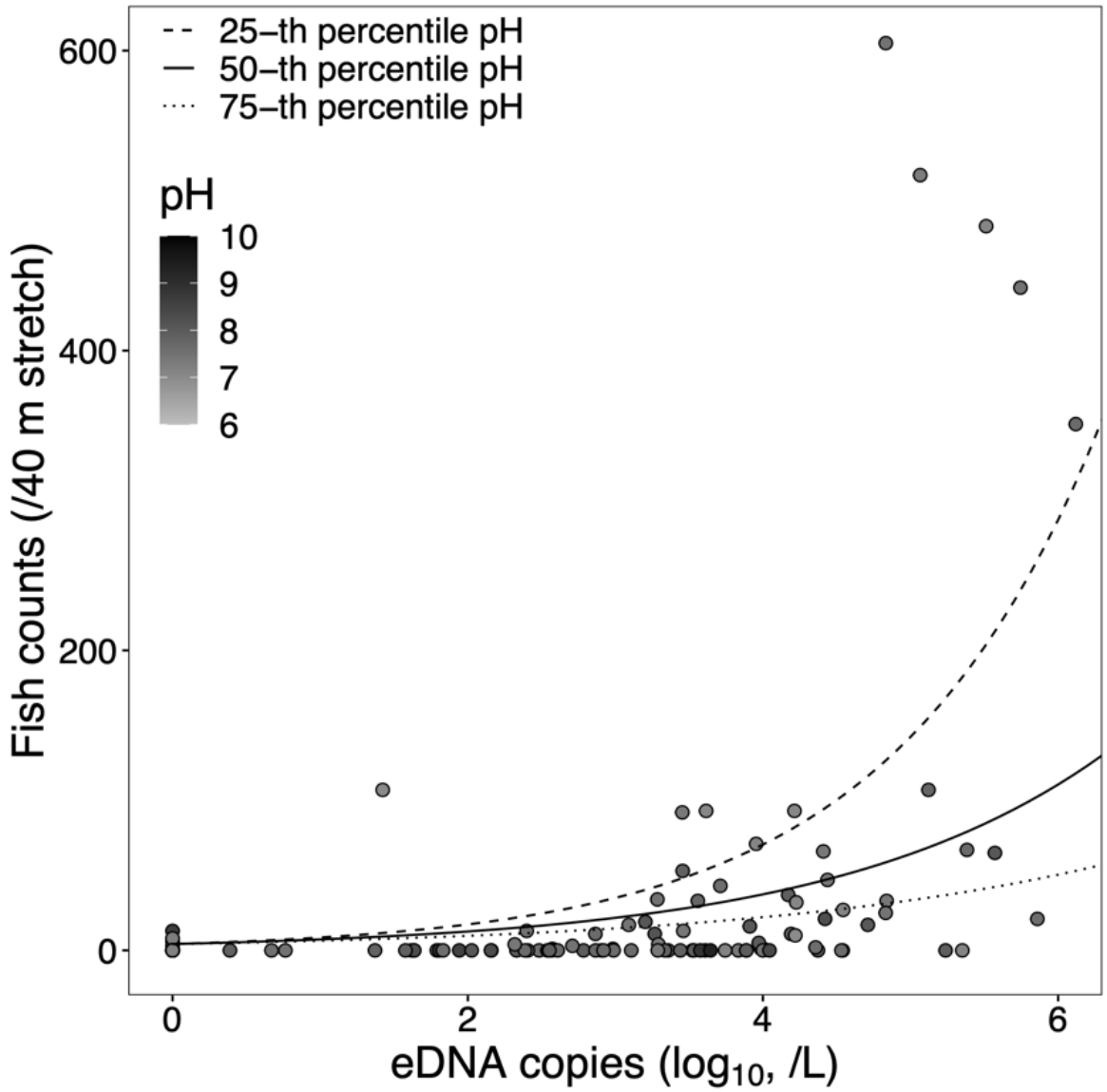

Figure 2.3. Relationship between eDNA copies at sites within the river channel (log_{10}, /L) and fish counts from visual inspection (/40 m stretch) in the river channel, as revealed by the zero-hurdle model. dashed, solid, and dotted lines indicate the equations at the 25-th percentile pH (7.37), 50-th percentile pH (7.71), and 75-th percentile pH (7.99), respectively.

Table 2.2. Presence or absence of Hasu eDNA copies (determined through eDNA analysis) and presence or absence of Hasu fish (determined by visual inspection), evaluated in relation to environmental factors as explanatory variables using general linear models (GLMs) with coefficients (estimates ± standard errors) selected via stepAIC. For presence or absence of eDNA copies vs environmental factors, river mouth and river channel sites were assessed independently. For presence or absence of fish by visual inspection vs environmental factors, only datasets from the river channel sites were used since no visual inspections were conducted at the river mouth.

Coefficients	**Presence or absence of Hasu eDNA**		**Presence or absence of Hasu fish**
	River mouth	**River channel**	**River channel**
(Intercept)	$-4.93 \times 10^{0} \pm 2.15 \times 10^{0*}$	$-2.51 \times 10^{0} \pm 1.02 \times 10^{0*}$	$-1.79 \times 10^{0} \pm 5.18 \times 10^{-1***}$
Presence of gravel	$2.57 \times 10^{0} \pm 8.24 \times 10^{-1**}$		$1.22 \times 10^{0} \pm 4.85 \times 10^{-1*}$
Presence of sand	$1.85 \times 10^{0} \pm 7.93 \times 10^{-1*}$		
Presence of clay			
River width (m)	$8.61 \times 10^{-3} \pm 5.57 \times 10^{-3}$	$3.26 \times 10^{-2} \pm 1.38 \times 10^{-2*}$	$3.68 \times 10^{-2} \pm 1.40 \times 10^{-2**}$
River depth (m)			
Electrical conductivity (μS/cm)			$-4.29 \times 10^{0} \pm 2.49 \times 10^{0}$
Turbidity ($\log_{10}$, NTU)		$-2.31 \times 10^{-2} \pm 1.51 \times 10^{-2}$	$-9.10 \times 10^{-2} \pm 6.06 \times 10^{-2*}$
pH		$2.62 \times 10^{-1} \pm 1.32 \times 10^{-1*}$	
Water velocity ($\log_{10}$ (x+1), cm/s)		$9.81 \times 10^{-3} \pm 4.49 \times 10^{-3*}$	$8.91 \times 10^{-3} \pm 5.02 \times 10^{-3}$
Water temperature (°C)	$1.69 \times 10^{-1} \pm 7.73 \times 10^{-3*}$		

Table 2.3. Positive observations (greater than 0) of Hasu eDNA copies (determined through eDNA analysis) and positive visual counts (greater than 0) of Hasu fish (determined by visual inspection), evaluated in relation to environmental factors as explanatory variables using simple linear models (LMs) with coefficients (estimates ± standard errors) selected via stepAIC. For positive number of eDNA copies vs environmental factors, river mouth and river channel sites were assessed independently. For individual counts vs environmental factors, only datasets from river channel sites were used since no counts by visual inspection were obtained at the river mouth.

Coefficients	Positive Hasu eDNA copies (log_{10}, copies/L)		Positive visual counts (log_{10}, /40 m)
	River mouth	River channel	River channel
(Intercept)	$4.96 \times 10^{0} \pm 1.54 \times 10^{0**}$	$7.38 \times 10^{0} \pm 2.03 \times 10^{0***}$	$5.82 \times 10^{0} \pm 1.95 \times 10^{0**}$
Presence of gravel	$6.02 \times 10^{-1} \pm 1.72 \times 10^{-1***}$	$5.35 \times 10^{-1} \pm 2.62 \times 10^{-1*}$	
Presence of sand	$3.36 \times 10^{-1} \pm 2.01 \times 10^{-1}$		
Presence of clay			
River width (m)			$6.06 \times 10^{-1} \pm 2.46 \times 10^{-1*}$
River depth (m)		$6.07 \times 10^{-1} \pm 3.37 \times 10^{-1}$	
Electrical conductivity (μS/cm)	$-4.79 \times 10^{0} \pm 1.51 \times 10^{0**}$		$-2.36 \times 10^{0} \pm 1.34 \times 10^{0}$
Turbidity (log_{10}, NTU)		$-1.01 \times 10^{0} \pm 2.98 \times 10^{-1**}$	
pH	$-2.62 \times 10^{-1} \pm 1.80 \times 10^{-1}$	$-5.50 \times 10^{-1} \pm 2.65 \times 10^{-1*}$	$-5.89 \times 10^{-1} \pm 2.62 \times 10^{-1*}$
Water velocity (log_{10} (x+1), cm/s)	$6.60 \times 10^{-1} \pm 2.71 \times 10^{-1*}$		
Water temperature (°C)			

2.4.3 Effect of environmental factors on Hasu eDNA copies and visual counts

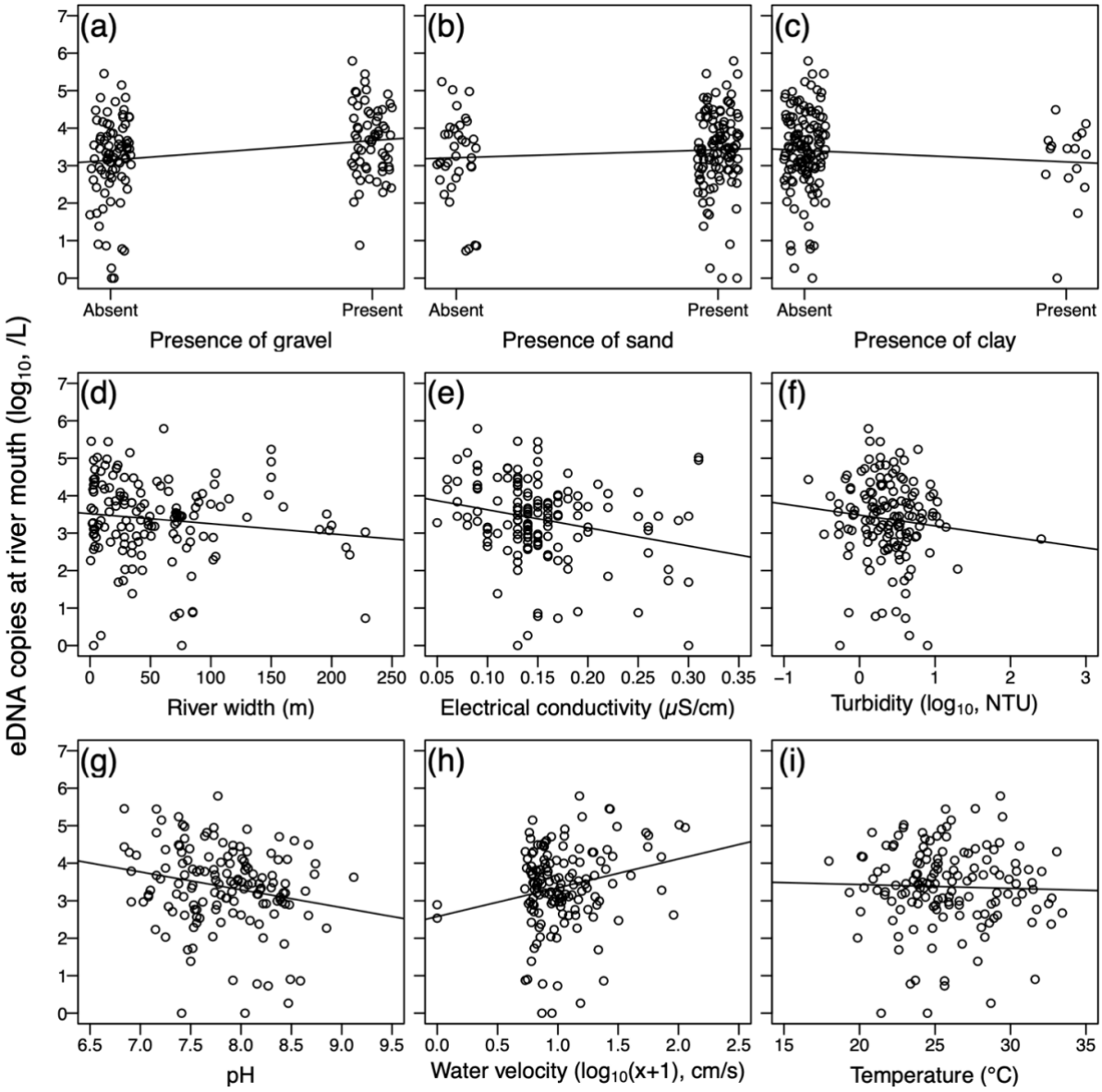

Figure 2.4. Panels (a)–(i) indicate plots of positive eDNA observations (log10, copies/L) as the response variable and environmental factors as explanatory variables at the river mouth sites. For the presence of gravel (a), sand (b) and clay (c), the binomial variables were jittered to reduce dense clustering of points during plotting.

At the river mouth, using stepAIC in the GLM model, we identified gravel and sand presence, river mouth width, and water temperature as factors influencing the presence of eDNA copies at sampling sites (Table 2.2). However, only gravel and sand presence, along with temperature, showed significant positive effects ($p < 0.05$). Additionally, in the linear model, gravel presence in the river and water current velocity positively influenced the number of

eDNA copies obtained at sampling sites, while electrical conductivity had a negative impact (Table 2.3, Figure 2.4, Adj. R^2: 0.18, $p < 0.05$). At the river channel sites, using stepAIC, we identified river width, turbidity, pH, and current velocity as factors influencing the presence of eDNA at sampling sites (Table 2.2). Nevertheless, all factors, except for turbidity, had positive and significant effects in the assessment ($p < 0.05$). In the second part of the analysis, only gravel presence had a positive and significant influence on the model, while pH and water turbidity had negative but significant impacts on the number of eDNA copies obtained at the sampling sites (Table 2.3, Figure 2.5, Adj. R^2: 0.16, $p < 0.05$).

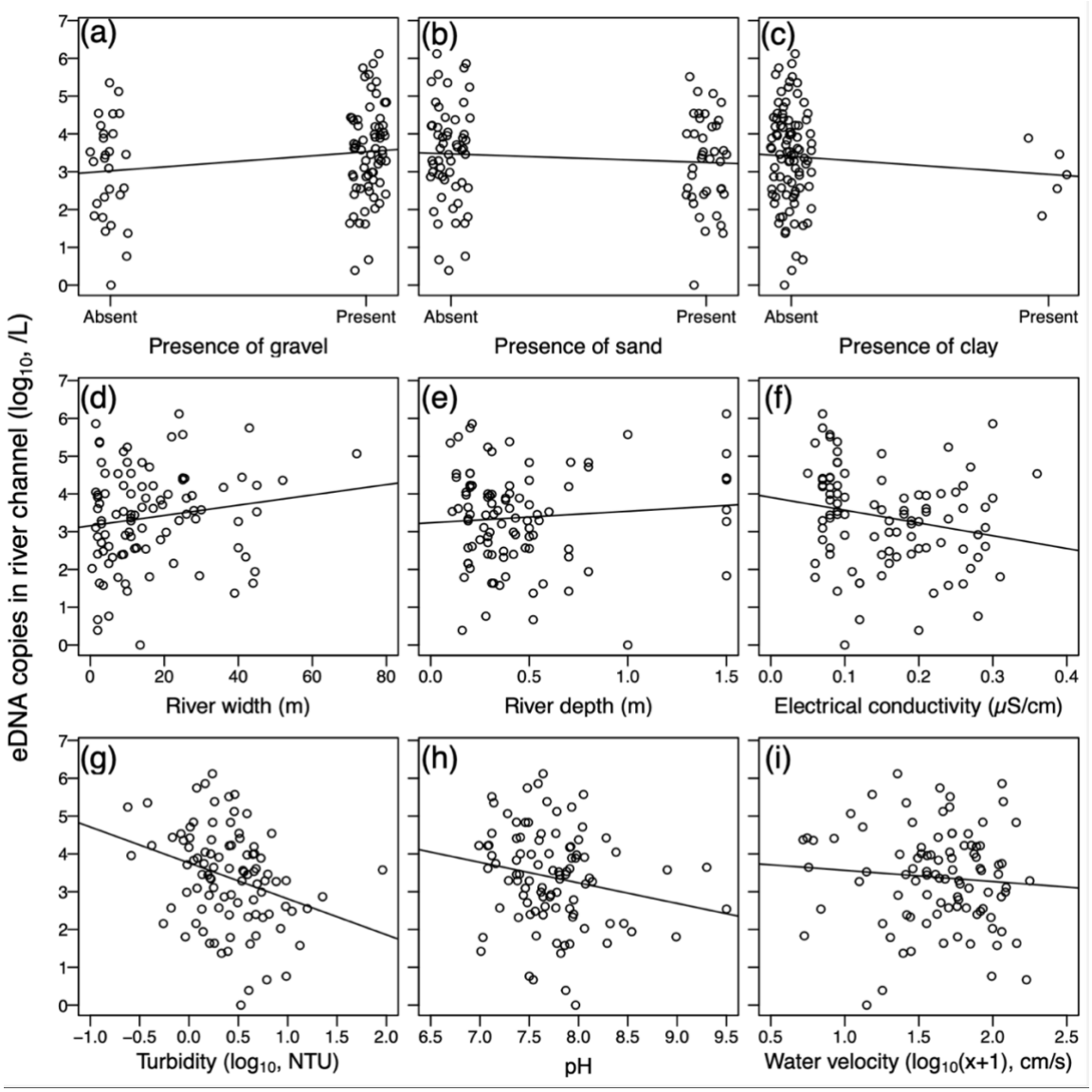

Figure 2.5. Panels (a)–(i) indicate plots of positive eDNA observations (log10, copies/L) as the response variable and environmental factors as explanatory variables within the river channel. For the presence of gravel (a), sand (b) and clay (c), the binomial variables were jittered to reduce dense clustering of points during plotting.

For the presence of Hasu fish at river channel sites, using stepAIC, we identified gravel presence, river width, electrical conductivity, turbidity, and current velocity as factors influencing fish presence (Table 2.2). However, only gravel presence and river width had a positive and significant effect in the assessment, while turbidity had a negative but significant impact on the model ($p < 0.05$). In the linear model assessing the impact of environmental factors on visual counts, only river depth had a positive effect on the model, while pH had a negative but significant impact on the number of Hasu fish at the sampling sites (Table 2.3, Figure 2.6, Adj. R^2: 0.21, $p < 0.05$).

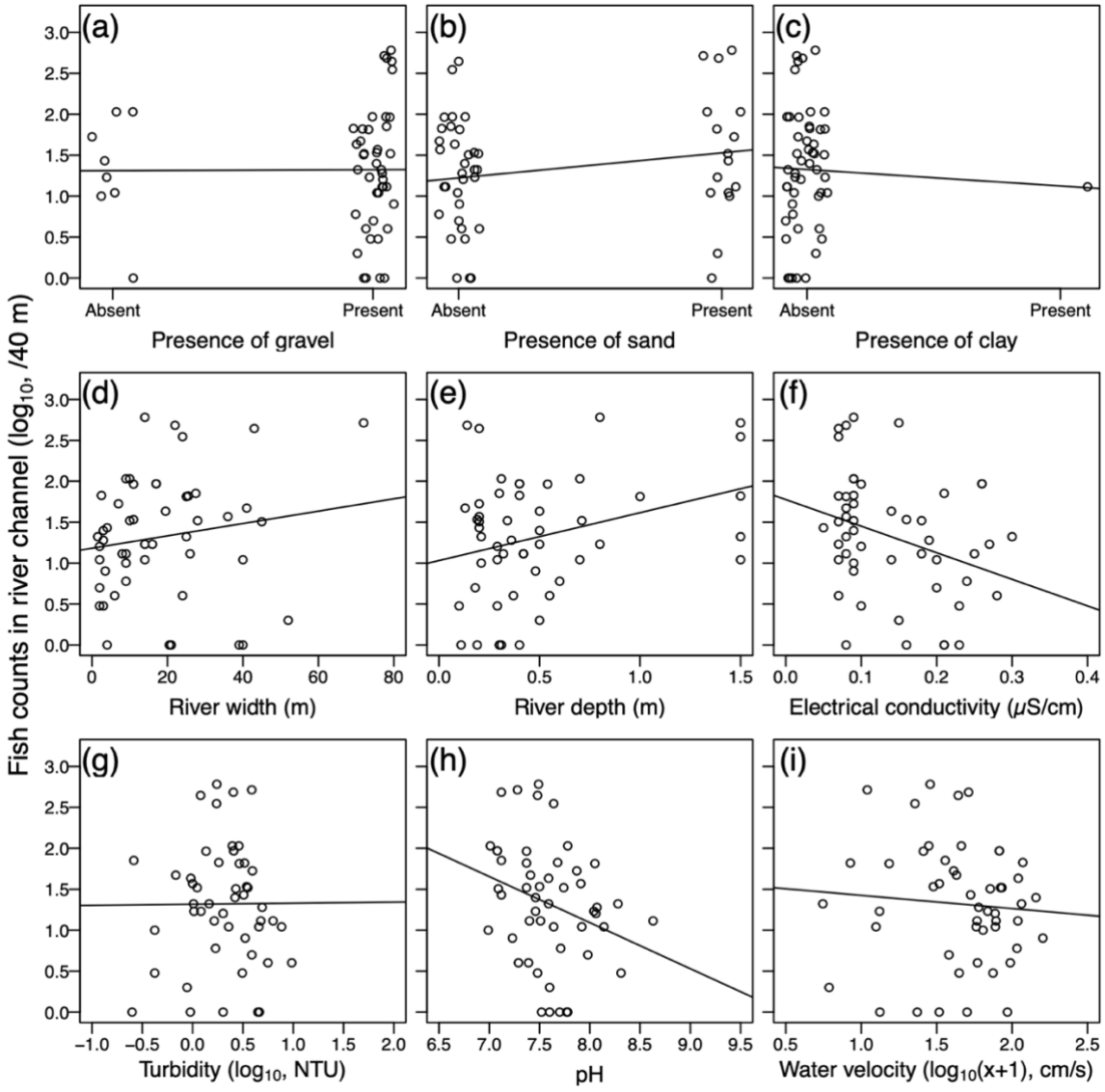

Figure 2.6. Panels (a)–(i) indicate plots of positive visual encounters (log10, counts/40 m) as the response variable and environmental factors as explanatory variables within the river channel. For the presence of gravel (a), sand (b) and clay (c), the binomial variables were jittered to reduce dense clustering of points during plotting.

2.5 Discussion

2.5.1 Significance of using complementary methods on Hasu fish migration ecology

We found positive correlation between the number of Hasu eDNA copies and counts through visual inspection (Figure 2.3, Table 2.1). Maruyama et al. (2018) previously demonstrated a similar positive correlation between the number of Hasu eDNA copies and counts through visual inspection. However, due to the limited sample size, specifically data obtained from a single river, Maruyama et al. (2018) were unable to statistically account for the effect of environmental factors on this relationship. By using a zero-hurdle model and data from the 32 rivers, this study was able to account for the effect of environmental factors on the relationship between Hasu eDNA copies and counts through visual inspection. Thus, the number of migrating Hasu individuals in the 32 river channel sites, within a 40 m stretch upstream from an eDNA sampling point, can be best estimated using eDNA copy numbers ($\log_{10}$) from a one-liter sample and the interaction term between the eDNA copy numbers ($\log_{10}$) and the pH of the water sample. This can be expressed as an equation as follows:

$$N_{Hasu} = 4.44 \times log_{10}\,[eDNA] + 0.47 \times log_{10}\,[eDNA] \times pH - 2.83$$

Equation 2.1. Estimating the number of Hasu spawning individuals within a 40-m stretch from an eDNA water sample in Lake Biwa tributaries derived from the zero-hurdle model. Where: N_{Hasu} is the number of estimated Hasu fish in the 40-m stretch upstream of an eDNA sampling point; [eDNA] is the number of eDNA copies in a liter of the river water sample and pH is the pH of the water at the sampling site.

The introduction of pH into the relationship between Hasu eDNA copies and counts through visual inspection represents progress in the fields of eDNA analysis and assessment tools for identifying Hasu fish migration hotspots. However, if not carefully considered, it could lead to an underestimation of the number of migrating Hasu fish in the rivers. In this study, the average pH in the river channel sites was high at the beginning of the reproductive season, i.e., in May (mean pH = 8.32 ± 0.54) and decreased with progression of the reproductive season, with the lowest pH being recorded in August (mean pH = 7.53 ± 0.40), before slightly increasing again at the end of the reproductive season, i.e., in September (mean pH = 7.66 ±

0.31). As the pH increases, the relationship curve between eDNA copies and counts by visual inspection flattens (Figure 2.3, Table 2.1) and moves closer to zero. Therefore, at high pH levels, estimating individual counts from eDNA copies becomes difficult, leading to increased uncertainty and possibly underestimation.

Although DNA is relatively stable in alkaline solutions, it may denature at extremely high pH because some hydrogen bond acceptors involved in base pairing become protonated (Moret et al. 2001). It is also worth mentioning that this study used glass fiber filters to collect eDNA from water samples, which have been documented to have low collection efficiency in alkaline solutions (Tsuji et al. 2017). This could result in an underreporting of the amount of eDNA present in the river and affect results. When possible, using complementary methods, such as counts through visual inspection, in highly alkaline rivers and at the beginning of the reproductive season when pH levels are generally high, causing the eDNA-pH based model to become unreliable, could help address the issue of underestimation and provide more accurate estimates of the number of Hasu fish migrating in the rivers.

In some rivers, especially at the river mouth, visual inspection was difficult due to factors such as high turbidity, increased depth, greater width, and the absence of bridges. In these rivers, eDNA analysis provided a more effective tool for detecting the presence or absence of migrating Hasu fish, primarily because of the ease in collecting water samples. The use of complementary methods in this study provided a holistic approach to better understand the migration patterns of Hasu fish in the 32 tributaries of Lake Biwa. The choice on which method to use at a sampling point rests entirely on the researcher's judgement. When using complementary methods, considering characteristics of the rivers and resources available to the researcher can help determine the most suitable method to use for Hasu fish migration assessment.

2.5.2 Migration hotspots of Hasu fish during the reproductive season and their characteristics

This study revealed that Hasu fish are widely distributed in the inlet tributaries of Lake

Biwa during the reproductive season, with a greater presence in tributaries on the north-western side of Lake Biwa. Both eDNA analysis and visual counting, showed that Hasu fish were abundant in rivers that had gravel or sandy substrates, were fast flowing at the river mouth, were less turbid, had reduced alkalinity, and were deep and wide enough to accommodate migrating individuals. These findings are consistent with previous observations by Tanaka (1964) and a study along the shoreline of Lake Biwa by Imamura (2018). The largest migrating groups of Hasu fish were recorded in Chinai and Ado rivers by visual counts and eDNA copy numbers, respectively (Figure 2.2). Both rivers are located on the northern-western side of Lake Biwa. This region has fewer human settlements and is characterized by mountain ranges relatively close to the lake compared to the eastern side. The Ado River, which has two arms feeding into Lake Biwa, had more migrations in the northern arm than in the southern arm. Additional assessment of the two river arms is needed to understand the variation in Hasu fish densities.

Ado River, Chinai River, Seri River, and Shiotsuo River, all located in the northern basin of Lake Biwa, exhibited similar trends in both eDNA copy numbers and visual counts. In these rivers, eDNA copies and visual counts increased from May to July during the reproductive season and decreased from August to September. This distinct change in fish counts and eDNA copies, with progression of the reproductive season, is indicative of recently migrating Hasu fish. Ado, Chinai, Seri, and Shiotsuo rivers are characterized by gravel and/or sandy substrates, do not dry out during the reproductive season and are sufficiently deep for upstream migration. These rivers, with their specific characteristics, are important for Hasu fish migration and can be considered as Hasu fish migration hotspots. In contrast, Hasu fish were not visually observed in most rivers in the southern basin. No eDNA and visual counts were obtained at the river channel sites in Hira River, Otani River, Omiya River, Yana River, Sagami River, and Kusatsu River. Several factors contribute to these observations, including drying up during the reproductive season in some rivers (e.g., Otani, Hira, and Omiya rivers), inadequate depth to

accommodate Hasu upstream migration in others (e.g., Sagami and Yana rivers), and possibly the redirection and modification of these rivers to meet human needs in recent history (e.g., Yogo river in the northern basin and Kusatsu river in the southern basin; Ministry of Land, Infrastructure, Transport and Tourism, Japan 2016a, 2016b). There was also little or no eDNA at the river mouth in Yana River, Sagami River and Kusatsu River. This implies that there were fewer or no Hasu fish approaching these rivers.

Compared to the north-western side, there are fewer Hasu migrations on the other sides of Lake Biwa, which are more urbanized, and the water quality is poorer, especially in the southern basin. This could be a result of agricultural inputs from surrounding paddy fields and high population density in the regions (Yoshioka 1991, Shiga Prefectural Government 2018). Extreme changes in pH due to agricultural inputs have been documented to create toxic environments for some freshwater fish elsewhere (Zhou & Boyd 2014). In addition, land reclamation in the 1960s has led to significant modifications on the coastline of Lake Biwa (Zeballos & Yamaguchi 2011). The water quality in Lake Biwa deteriorated significantly in the 1960s due to rapid population growth, inadequate wastewater treatment, and agro-chemical abuse, all of which are key drivers of eutrophication in lakes (Kita et al. 2006). Some rivers have been redirected, while others reconstructed to accommodate human needs. Reconstruction has also seen the introduction of concrete riverbanks and substrates in some rivers (e.g., in Sagami River), prompting the reduction of gravel, a vital component for Hasu fish reproductive migration. Despite efforts by the local government to prevent further deterioration of the lake's ecosystem, ecosystem restoration has been slow—potentially explaining the fewer migrations in this region. Changes in land use and artificial modifications to rivers have the potential to reshape sediment distribution and flow velocity. Rivers that flow from the highly developed regions, such as those in the southern basin, tend to have smaller downstream gradients compared to those in less developed regions, like those the northern basin (Weilhoefer et al. 2022). It is possible that Hasu fish avoided migrating to the rivers with smaller downstream

gradients, as our results show that Hasu fish tend to prefer rivers with sandy-gravel substrates and fast-flowing water.

Although visual confirmation was not possible in some turbid rivers, such as the Shiratori River located on the eastern side of the northern basin, the presence of eDNA copies indicated recent Hasu fish migrations—albeit in smaller quantities than in clear running rivers. Most piscivorous species, including Hasu fish, rely on their keen eyesight for foraging (Pita et al. 2015, Hori et al. 2021). Turbid waters, as those in Shiratori River, may affect the line of sight in Hasu fish, making it difficult to forage for prey species. Furthermore, piscivorous species like Hasu fish require higher oxygen levels due to their quick foraging movements (Jackson et al. 2011, Hansen et al. 2012). The presence of particles in murky and turbid rivers may obstruct oxygen intake in their gills, causing Hasu fish to avoid such rivers. If the population of Hasu fish is to be increased in Lake Biwa and its ecosystem, there is a need to protect rivers with clear running water, especially rivers on the western side of the northern basin and improve the quality of water especially in the southern basin. However, further assessment is needed to understand the impacts of turbidity on prey capture in chasing fish such as Hasu.

In conclusion, this study advocates using complementary methods for least studied species such as Hasu fish. Our findings revealed the distribution of the potamodromous Hasu fish and its eDNA during its reproductive migration in 32 tributaries around Lake Biwa. The study also highlighted the significance of incorporating environmental factors such as pH into Hasu fish migration studies. Our eDNA analysis and visual count data revealed that Hasu fish migration occurs mostly in the northern basin of Lake Biwa especially in the western side. There is a need to protect rivers in this region, including rivers like the Ado River, Chinai River and Shiotsuo River, to name a few, during the reproductive season. These rivers have good water quality, fast-flowing currents, sandy-gravel substrates, are wide and deep enough to accommodate migrating Hasu fish and do not dry up, all of which are important for successful reproductive migration of Hasu fish.

3 Biometrics of migrating Hasu fish and exploring differences in the timing of upstream migration using stable isotopes

3.1 Chapter summary

I explored the biometrics of migrating Hasu fish to enhance our understanding of how the species has changed since the last extensive study by Tanaka in the 1960s. I also explored the differences in timing of upstream migration by using stable isotope ratios in Hasu tissue. Hasu fish were collected monthly, from May to September 2019, using cast nets. The biometric measurements: wet weight, standard length, gonad weight and gut content were collected and used to calculate the gonado-somatic index (GSI), Fulton's condition constant (K) and determine the feeding habits of Hasu fish. Carbon and nitrogen isotope ratios ($\delta^{13}C$ and $\delta^{15}N$ isotopes) in slow changing muscle and fast changing mucus tissues of Hasu fish were also used to determine recent diet change. At the beginning of the reproductive season, fewer females than males were caught, however the number of females increased as the season progressed. On average, males were larger than females. Migrating individuals were healthy ($K > 1$) and over 37% larger in the size than those in the 1960s. Gut content analysis revealed, for the first time, Hasu fish feeding in the rivers, primarily on Ayu fish, during the reproductive migration. $\delta^{13}C$ and $\delta^{15}N$ in muscle and mucus indicated a recent change in diet, i.e., from Lake Biwa to Shiotsuo River, with differences in the onset of feeding (and consequently upstream migration) between sexes and individuals. For the effective conservation of Hasu fish in the other tributaries where Ayu fish traps block other fishes' migration, I recommend having the rivers open from June to September to cover a range wide enough to accommodate the variable timing in upstream migration of Hasu fish.

3.2 Introduction

Opsariichthys uncirostris uncirostris (Hasu fish), is a potamodromous fish that relies on lake-river migration for its reproduction. It is the only piscivorous cyprinid fish in Japan,

endemic as a sub-species to Lakes Biwa and Mikata. Its population has been experiencing a continued decline for the last 70 years and it is considered vulnerable in Lake Biwa and extinct in Lake Mikata (Ministry of the Environment, Japan 2020). Unfortunately, the dynamics surrounding Hasu fish reproductive migration has not been extensively studied since the 1960s. The fish is reported to spawn in summer from late May to early August in the shores of Lake Biwa and its tributaries, with mostly mature males and females of ages 3 years (average body length: 160 mm) and 2 years (average body length:130 mm), respectively (Miura 1966, Tanaka 1964). Using environmental DNA (eDNA) analysis and counts through visual inspection, Maruyama et al. (2018) and Mvula et al. (2023) were able to determine that Hasu fish abundance gradually increases in tributary rivers of Lake Biwa during the reproductive season from May to July before eventually decreasing in August and September. Even though eDNA provides a snapshot of the distribution of Hasu fish in Lake Biwa tributaries, it currently does not offer insights into intake of nutrients and residence time of fish in the tributaries. These can be estimated through the feeding habits and biometrics of the migrating fish. However, the feeding habits of Hasu fish during its reproductive migration to Lake Biwa tributaries are currently unknown. These feeding and biometric assessments are important to determine the overall health status and thus aid in the conservation of Hasu fish during reproductive migration.

For decades, conventional measurements, including diet assessments and length-weight measurements, have been the gold standard for gaining insights into the feeding habits and biometrics of various migrating species. For instance, wet weight and standard-length measurements have been used to provide data on individual fish size and mass, enabling us to monitor the growth and overall condition of a species (Moutopoulos & Stergiou 2002). Analyzing gut contents has also allowed for a better understanding of the feeding behavior and dietary preferences of such species during migration. In addition, indices such as the gonado-somatic index (GSI) and Fulton's condition constant (*K*) have been used to assess the timing and intensity of spawning events (Mozsár et al. 2015, Roy et al. 2014). These biometric tools

can thus be used to reflect the overall health status of migrating Hasu fish and consequently aid in the conservation of the fish in Lake Biwa tributaries.

In recent years, the use of stable isotopes has also gained traction in ecological studies. A stable isotope is an element whose relative proportions do not vary over time due to the absence of radioactivity (Gill 2015). Stable isotopes remain unchanged and transfer in a predictable manner between trophic levels (Hobson et al. 1997). When a species migrates and begins to feed in a new environment, the composition of stable isotopes within its tissues changes, reflecting the isotope ratios of the diet in the new environment (Phillips & Eldridge 2006). Different tissues have different turnover rates, i.e., the rate at which the tissues change to reflect the isotope ratios in the new environment (Hobson & Clark 1992). For example, in fish, mucus tissues tend to have faster turnover rates when compared to dorsal muscle and fin tissues (Maruyama et al. 2016, Shigeta et al. 2017, Winter et al. 2019).

By taking advantage of the differences in turnover rates in the two tissues (e.g., the slow change in muscle and the fast change in mucus tissues), and on the likely assumption that the migrating animals are in isotopic equilibrium with its former environment when they start migration, migration researchers can roughly estimate the time when a species changed its diet, and consequently its environment from one sampling (Phillips & Eldridge 2006, Heady & Moore, 2013). It is important to emphasize that the environmental equilibrium values may vary between tissues due to the inherent differences in stable isotopes and their specific diet-tissue discrimination factors (i.e., the differences between a tissue's stable isotope ratio and that of its diet when in isotopic equilibrium). For instance, using adult *Silurus asotus* as a model species in a diet switch experiment, Maruyama et al. (2016) demonstrated that the initial stable carbon isotope ratios were higher in muscle tissue than in mucus tissue. Regardless of the differences in equilibrium values and even though stable isotope ratios in the tissues change at different rates, it is assumed that they (stable isotope ratios in different tissues) are bound to reach new equilibriums with a consistent diet in the new environment. The two commonly used stable

isotopes in ecological studies are carbon and nitrogen isotope ratios (hereafter, $\delta^{13}C$ and $\delta^{15}N$, respectively), which exhibit a stepwise increase of about 1‰ and 2.5 – 5‰ at each trophic level, respectively (DeNiro & Epstein 1978, DeNiro & Epstein 1981, Minagawa & Wada 1984, Hobson et al. 1997, Post 2002). These two stable isotopes play a crucial role in understanding the transfer of carbon and nitrogen through the food web in different ecosystems.

Generally, lake ecosystems tend to have lower $\delta^{13}C$ and higher $\delta^{15}N$ when compared to river ecosystems (Le Bourg et al. 2018, Wilkinson et al. 2022). This is largely due to the differences in reaction rates between phytoplankton and periphyton. In the Lake Biwa ecosystem, in particular, $\delta^{13}C$ have been documented to be higher in tributary rivers than in the lake, with zooplankton in the lake exhibiting lower $\delta^{13}C$ than benthic invertebrates in the tributaries (Yamada et al. 1998, Maruyama et al. 2001, Sawada et al. 2019). Using eggs in ovaries of fish, Ito et al. (2015) and Sawada et al. (2019) also independently demonstrated that $\delta^{15}N$ are higher in Lake Biwa than in its tributaries, reflecting the differences between primary producers and basal animals (Yamada et al. 1998). Thus, it is expected that Hasu fish migrating from Lake Biwa and feeding in the tributaries will exhibit $\delta^{13}C$ and $\delta^{15}N$ changes in its tissues to reflect those of the river environment over a period of time.

By integrating data from the conventional biometric measurements and stable isotope ratios, we can develop a more comprehensive portfolio on Hasu fish populations during their reproductive migration. Therefore, the aim of this study was to assess the feeding habits and residential time of migrating Hasu fish, in a Lake Biwa tributary, by using conventional biometric measurements and examining stable isotope ratio changes in its mucus and muscle tissues across its reproductive season. This integrated approach should facilitate conservation efforts and the adoption of sustainable practices to ensure the long-term viability of Hasu fish and the preservation of its ecosystem.

3.3 Materials and Methods

3.3.1 Description of study site

The study was conducted in the lower reaches of the Shiotsuo River, flowing into Lake Biwa from the north. The river has a total length of 9 km and a basin area spanning 21.8 m^2 (Ministry of Environment, Japan 2023). The river primarily flows through mountainous terrain (95.8% of the total river catchment area), contributing to a steep gradient of 9.5 m/km, making it one of Lake Biwa's steepest rivers and a preferred reproductive upstream migration route for Hasu fish. Due to its steep gradient, the river is fast-flowing, and it is also characterized by gravel bottom substrates, all of which have been identified to be key drivers of Hasu reproductive migration (Chapter 2). In addition to the ease of sample collection, these characteristics are why Shiotsuo River was selected as a study site in this study.

The river is perennial, and there are no high weirs in the middle and lower reaches of the river, allowing for natural upstream migration of Ayu fish, as well as Hasu fish. Ayu fish serves as the primary food source for Hasu fish in Lake Biwa (Tsunoda et al. 2015), and possibly in the tributaries, but the feeding habits of Hasu fish in rivers during reproductive migration remains unknown. Abundant natural Ayu fish in the river also attracts recreational fishermen, but fishing is not permitted in the lower reaches, where Ayu spawning parents are released approximately 1 km upstream from the river mouth. The river also has a protected spawning area that extends 4.5 km from the river mouth. These characteristics make Shiotsuo river a conducive environment for Hasu fish reproductive upstream migration.

3.3.2 Fish sampling and biometric measurements

Hasu fish samples were collected monthly in the lower reach (2–3 km from the river mouth) of Shiotsuo River from May to September 2019 using cast nets. The sampling was systematic and a target of 20 Hasu individuals was set for each sampling. Wet weight (g), standard length (mm), gonad weight (g) were measured (to the nearest 0.01 g or 0.1 mm) in the field. The species composition of the gut content was also recorded in the field. The gut contents

of each fish, and the fish itself, were put in individual Ziploc® bags to avoid inter-sample contamination and kept on ice in a cooler box while in the field. After the field sampling, samples were transported to the laboratory at Ryukoku University and kept frozen below –22.5°C until analysis. The Fulton's condition constant (*K*) and gonado-somatic index (GSI) were also calculated for each fish using the following formulas, respectively:

$$K = 100 \times \frac{W}{L^3}$$

Equation 3.1. Equation to calculate Fulton's condition constant (K). Where: W (g) is the weight of the fish and L (cm) is the length of the fish.

$$GSI = 100 \times \frac{W_g}{W_b}$$

Equation 3.2. Equation to calculate the gonado-somatic index. Where: W_g (g) is the gonad weight and W_b (g) is the body weight of the fish.

3.3.3 Multi-tissue stable isotope analysis

At the laboratory, epidermal mucus was wiped directly along the lateral line on the left body surface of each thawed specimen using quarter of a 25 mm diameter GF/F glass microfiber filter (GE Healthcare, Buckinghamshire, UK). Each filter was oven dried at 60°C for 48 h. and cleaned using forceps to remove scales or skin fragments if present (as done in previous studies e.g., Maruyama et al. 2016, Shigeta et al. 2017). Lipid extraction or mathematical corrections for mucus samples were not performed because glycoprotein and non-lipid components dominate the composition of mucus (Shephard 1994). After mucus sampling, dorsal muscle tissue was extracted from above the lateral section on the left side of same individual. Muscle tissue was also oven dried at 60°C for 48 h, ground to a fine powder and the effect of variable lipid content on $\delta^{13}C$ values corrected using the C:N ratio of each sample according to a fish general correction model by Post et al. (2007).

Stable isotope analysis was 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. Standards alanine [$\delta^{15}N$, 1.6 ± 0.2 ‰ (mean ± SD); $\delta^{13}C$, –19.6 ± 0.2 ‰ (mean ± SD)] and histidine [$\delta^{15}N$, –7.6 ± 0.2 ‰; $\delta^{13}C$, –10.7 ± 0.2 ‰] were used for calibration and quality control during the analysis through repeated measures. $\delta^{15}N$ and $\delta^{13}C$ were expressed as $\delta X = (R_{sample}/R_{standard}) - 1$, where X is ^{15}N or ^{13}C; R_{sample} is the $^{15}N/^{14}N$ or $^{13}C/^{12}C$ ratio of the measured samples; and $R_{standard}$ is the $^{15}N/^{14}N$ of atmospheric nitrogen or $^{13}C/^{12}C$ of Vienna Pee Dee Belemnite (VPDB). The analytical errors in the delta values were less than ± 0.3 ‰.

3.3.4 Data analysis and interpretation

All data analyses were conducted in R ver. 4.3.1 software. The Welch two sample t-test, base::t.test() with unequal variance in R, was used to compare the means of biometrics between all males (n = 33) and females (n = 24) in this study. On the other hand, non-parametric tests were used to assess the differences in stable isotope ratios between individuals across the sampling period and this was done in two steps: first, the non-parametric Kruskal-Wallis test, base::kruskal.test() in R, was used to assess whether the observed trends in isotope ratios, with the interaction between sex and sampling month as an explanatory variable, were significant. If the Kruskal-Wallis test produced a significant result, then a multiple comparison test using the Steel-Dwass test was conducted using the NSM3::pSDCFlig() with “Monte Carlo” as a method in the function due to the relatively small sample size in this study. The advantage of using Steel-Dwass test than conventional methods, such as the Dunnet method, is in its ability to solve multiple comparison problems more easily (Takagi et al. 2003). When statistically comparing males and females, only datasets from the July catch (n = 10 males, n = 10 females) and August catch (n = 9 males, n = 11 females) were used due to sample size limitations. The sample sizes in the June catch (n = 11 males, n = 1 female) and September catch (n = 3 males, n = 2 females) were not large enough to perform statistical comparisons. Furthermore, a separate analysis was conducted for male individuals comparing the June catch (n = 11 males), July catch (n = 10 males), and August catch (n = 9 males) due to a sufficient sample size for

statistical analysis.

The qualitative interpretation of multi-tissue isotope ratios was based on existing knowledge on $\delta^{15}N$ and $\delta^{13}C$. This approach was adopted due to the lack of specific turnover rates and trophic discrimination factors (TDFs) for $\delta^{15}N$ and $\delta^{13}C$ in Hasu fish tissues (fundamentally because this species' timid nature prevented them from smooth diet switch in our aquarium). We recognize the potential of the stable isotope clock, a method presented by Heady & Moore (2013), which takes advantage of the different turnover rates in two tissues to estimate the time since upstream migration (an analysis that would have been ideal for this study). Unfortunately, due to the unavailability of initial $\delta^{15}N$ and $\delta^{13}C$ for muscle and mucus tissues, along with the absence of specific turnover rates and TDFs, coupled with the inherently timid nature of the species, we were unable to directly estimate the time since upstream migration.

In closely related species, $\delta^{15}N$ and $\delta^{13}C$ tissue specific turnover rates are larger in mucus tissue than in muscle tissue (Maruyama et al. 2016, Shigeta et al. 2017, Winter et al. 2019). Thus, when $\delta^{15}N$ and $\delta^{13}C$ of populations with many immigrant individuals were compared, mucus tissue should exhibit greater variation than muscle tissues. Whereas such trend is obvious only for some time after migration because faster mucus tissue can saturate faster than the slow changing muscle tissue upon reaching isotopic equilibrium with the river environment after a long period of time, this saturation should not be observed in this study (half-life of mucus isotopic changes of three small cyprinid species were over two months; Shigeta et al. 2017). Theoretically, this variance is expected to indicate changes in the diet after movement of Hasu fish from the Lake Biwa to the Shiotsuo River, since riverine environments have higher $\delta^{13}C$ and lower $\delta^{15}N$ than lacustrine environments.

3.4 Results

3.4.1 Findings from the biometric measurements

No Hasu fish were caught in May despite our sampling efforts. In June, more male

individuals were caught than female individuals (Figure 3.1). In addition, the ratio of male to female Hasu individuals decreased with progression of the reproductive season. On average, the standard lengths (220.0 ± 26.3 mm (mean ± SD) for all males and 187.5 ± 22.8 mm for all females) and wet weights were larger in males than in females in this study (t = 4.98 and 4.86, $d.f.$ = 53.94 and 54.89, $p < 0.05$ in both tests). The gonad weight and gonado-somatic index were slightly larger in females than in males. However, only the gonado-somatic index was significantly different (t = -3.34, $d.f.$ = 39.52 and $p < 0.05$).

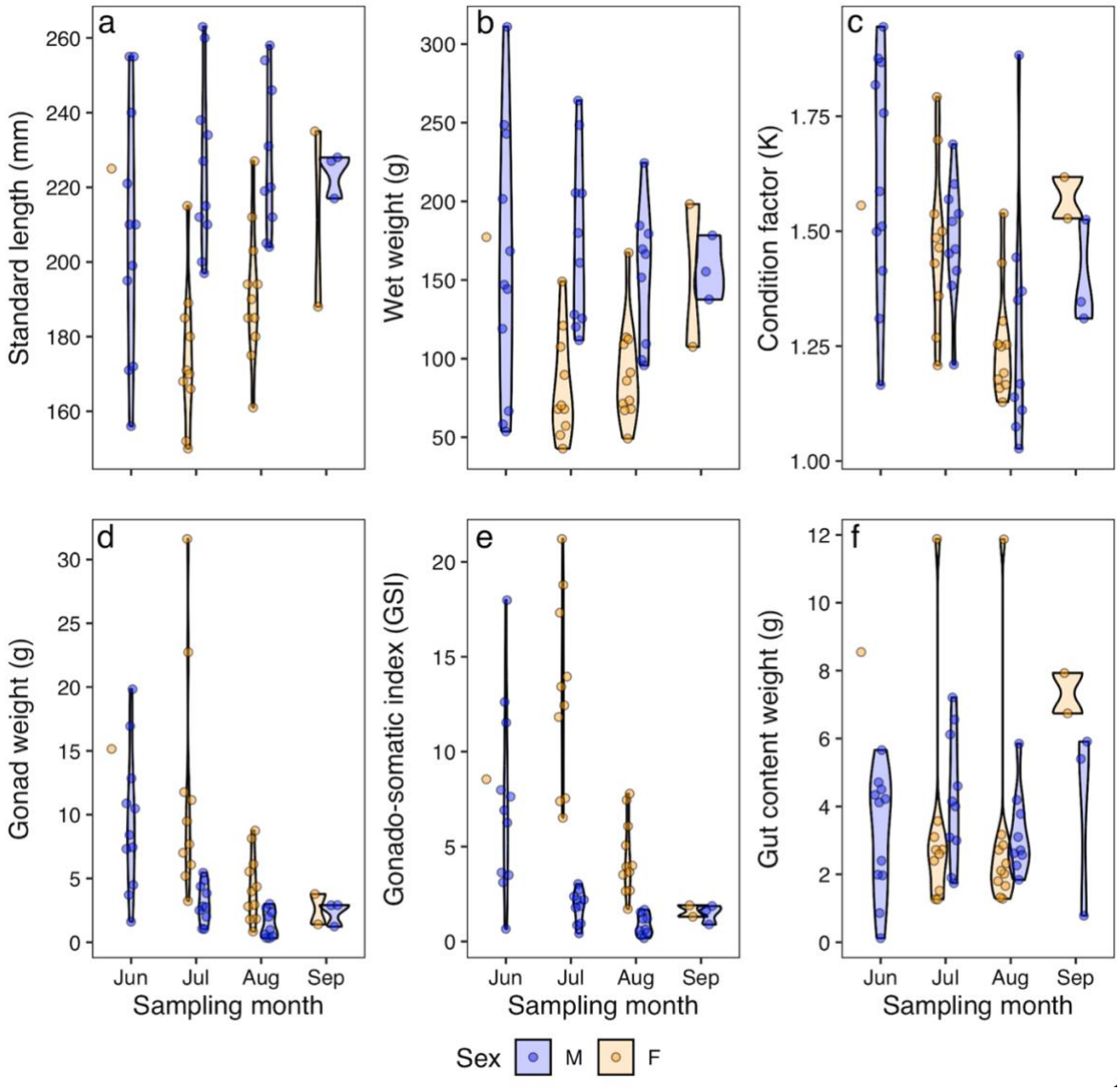

Figure 3.1. Changes in biometric measurements of Hasu fish during the reproductive season in Shiotsuo River: standard length (a), wet weight (b), Fulton's condition factor (c), gonad weight (d), the gonado-somatic index (e), and gut content weight (f). The blue and orange violin plots (with points) indicate male and female Hasu fish, respectively. The violin plots indicate the distribution of each measurement, where the width of the plot at any given point indicates density of the data. Violin plots and points were jittered to align

with each other. In May, no fish were caught despite sampling efforts; as a result, it was excluded from the plots.

In the June sampling, no food items were obtained from the guts of Hasu fish. However, the amount of gut content increased from July-August. The gut content analysis revealed that Ayu fish were the predominant food item of Hasu fish (Figure 3.2). Other food items like small insect appendages (e.g., legs) and egg-like structures were also obtained. From June to September, all caught individuals had a Fulton's condition factor (*K*) value of greater than one. The average *K* value decreased slightly in August before increasing again in September. It is also worth mentioning that there were no observable differences in the feeding frequency between males and females across the reproductive season.

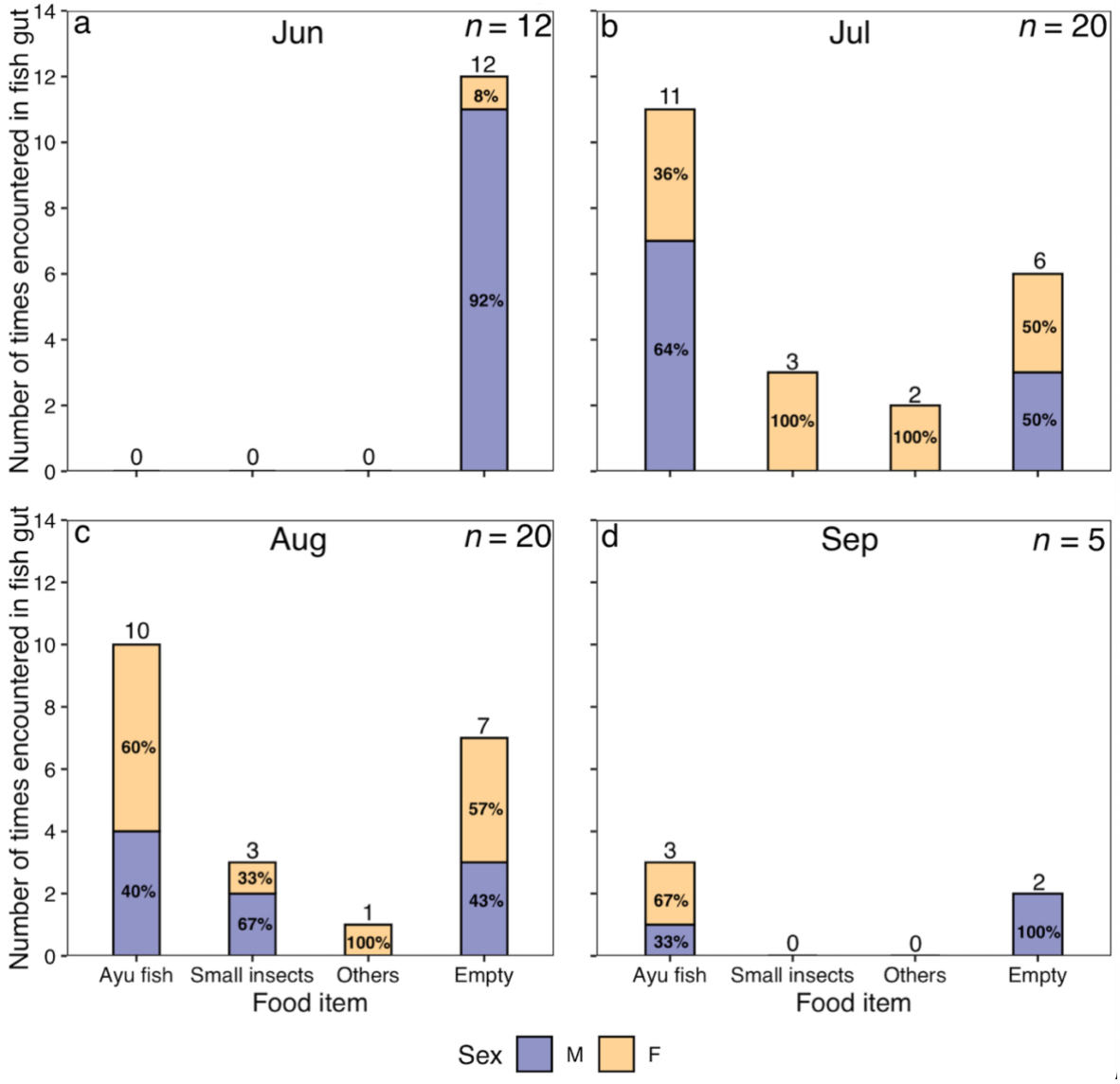

Figure 3.2. Number of times food items were encountered in the guts of male (blue) and female (orange)

Hasu fish caught in June (a), July (b), August (c), and September (d). In May, no fish were caught despite sampling efforts; as a result, it was excluded from the plots. The numbers on top of each bar indicate the number of times a food item was encountered in the guts of Hasu fish while the percentages (%) inside the bars indicate the proportion of male and female guts observed for each food item. The gut content of all fish were analyzed during each catch. In the plots, 'Others' included orange egg-like structures.

3.4.2 Findings from the multi-tissue stable isotope ratio analysis

There were some observable trends in $\delta^{13}C$ and $\delta^{15}N$ for muscle and mucus tissues for both males and females across the reproductive season (Figure 3.3). The slow changing muscle tissue was relatively unchanged across the reproductive season while the fast changing mucus gradually approached isotopic equilibrium with the river environment. However, the fast changing mucus never saturated. These trends in Hasu fish muscle and mucus tissues indicate a recent shift in diet from Lake Biwa to the Shiotsuo River, suggesting that the rate at which individuals reach isotopic equilibrium varies among individuals.

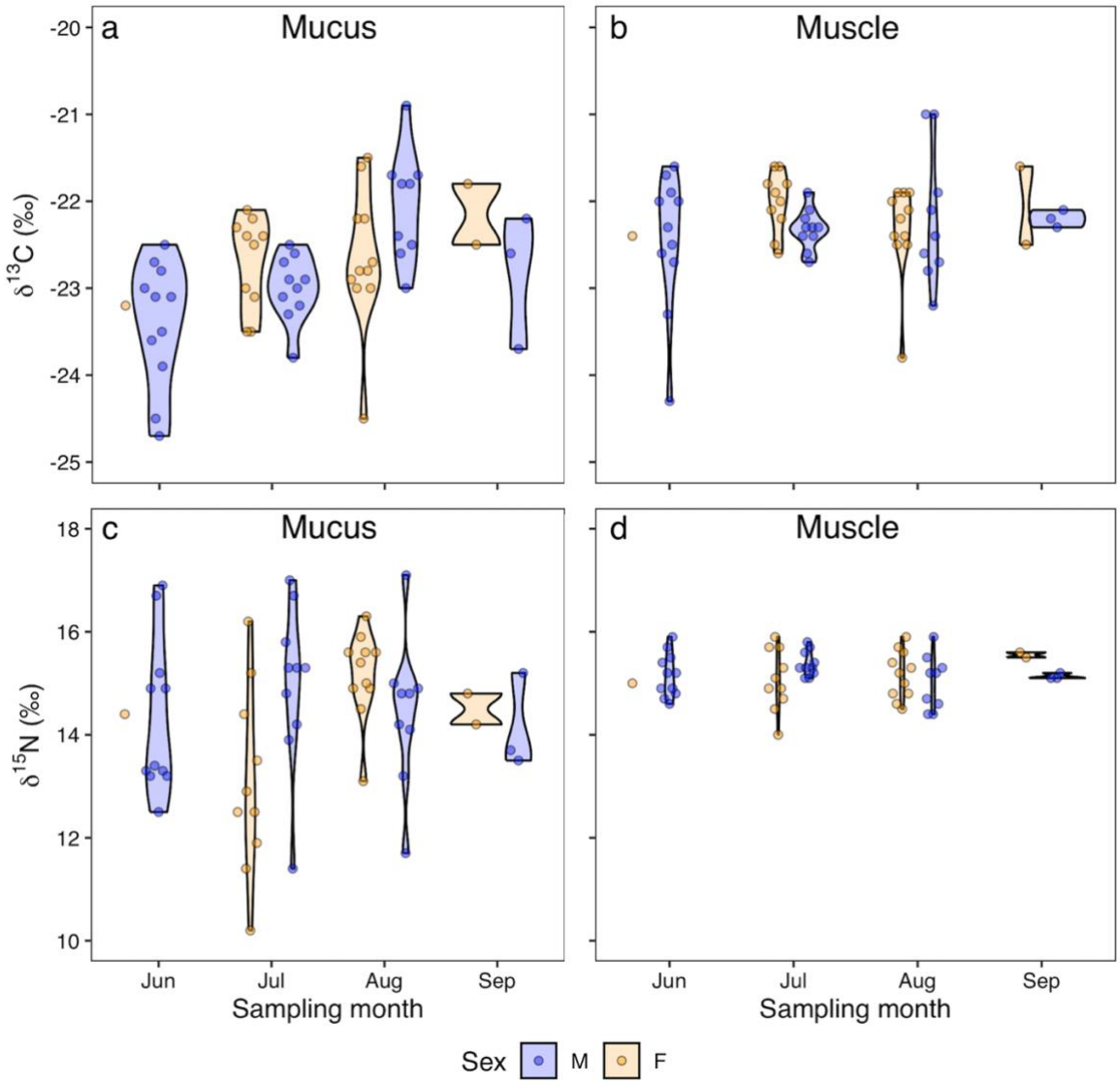

Figure 3.3. Changes in carbon (a and b) and nitrogen (c and d) stable isotope ratios (δ13C and δ15N, respectively) in mucus and muscle tissues of Hasu fish during the reproductive season in Shiotsuo River. The blue and orange violin plots (with points) indicate male and female Hasu fish, respectively. The violin plots indicate the distribution of stable isotope ratios, where the width of the plot at any given point indicates density of the data. Violin plots and points were jittered to align with each other. In May, no fish were caught despite sampling efforts; as a result, it was excluded from the plots. The change in $\delta^{13}C$ and $\delta^{15}N$ stable isotope ratios across the reproductive season reflects a change in Hasu fish diet from Lake Biwa to Shiotsuo River.

In the June sampling, the $\delta^{13}C$ were higher in muscle tissues than in mucus tissues for both males (n = 11) and females (n = 1). From June to July, there was an increase in the $\delta^{13}C$ for mucus in both males (n = 10) and females (n = 10) while the $\delta^{13}C$ in muscle remained relatively unchanged (Figure 3.3). This higher $\delta^{13}C$ in July suggests that more individuals were approaching isotopic equilibrium of the river (which is higher than that of the lake). From the

July to August catch, the $\delta^{13}C$ for muscle tissue was not significantly different between (Kruskal-Wallis chi-squared = 4.11, *d.f.* = 3, $p > 0.05$) males ($n = 9$) and females ($n = 11$) and the $\delta^{13}C$ appeared to be saturated. However, there was a significant difference in mucus $\delta^{13}C$ (Kruskal-Wallis chi-squared = 9.96, *d.f.* = 3, $p < 0.05$). Although the $\delta^{13}C$ for mucus tissue were lower than in females than those in the previous month, and while those in males increased, the comparison test revealed that the differences were only significant between males caught in July and males caught in August (W statistic = 4.34, $p < 0.05$).. When comparing males in the June catch, July catch and August catch, the Kruskal Wallis test also revealed significant differences in $\delta^{13}C$ of males caught in the 3 months (Kruskal-Wallis chi-squared = 14.72, *d.f.* = 2, $p < 0.05$). However, the differences were only significant in $\delta^{13}C$ of individuals caught in the July and those caught August (W statistic = 3.33, $p < 0.05$). This also indicated that the males were approaching isotopic equilibrium with the river environment earlier than the females.

Similarly, the $\delta^{15}N$ were higher in muscle than in mucus tissue for both males ($n = 11$) and females ($n = 1$) in the June sampling. From the June to July catch, there was no considerable difference, to the previous sampling, in $\delta^{15}N$ for muscle tissue in both males ($n = 10$) and females ($n = 10$) caught (Figure 3.3). There was also more variation in $\delta^{15}N$ ratios for mucus in both sexes, suggesting that there was feeding on items with different isotope signatures. From the July to August catch, the $\delta^{15}N$ for mucus tissues were slightly lower in males ($n = 9$) while those in females ($n = 11$) were higher in comparison to the previous month (Kruskal-Wallis chi-squared = 9.81, *d.f.* = 3, $p < 0.05$). The comparison test revealed that the differences were only significant between females caught in July and females caught in August (W statistic = 3.79, $p < 0.05$). The $\delta^{15}N$ in muscle tissues were not significantly different between males and females (Kruskal-Wallis chi-squared = 3.02, *d.f.* = 3, $p > 0.05$) and remained relatively unchanged across the sampling period, as is expected of slow turnover tissues. No significant differences were observed between the June catch, July catch and August catch in the male assessment of $\delta^{15}N$ (Kruskal-Wallis chi-squared = 2.16 and 2.95 for mucus and muscle tissues, respectively, *d.f.* =

2, $p > 0.05$). From the catches, some individuals had $\delta^{15}N$ that were closer to the equilibrium of the lake than the river, indicating of a recent change in diet from Lake Biwa to Shiotsuo River.

3.5 Discussion

3.5.1 New insights on the biometrics of Hasu fish and its feeding during reproductive migration

There was evidence of sexual dimorphism similar to a report by Tsunoda et al. (2023). Males exhibited larger standard lengths and wet weights than females with each catch. Hasu individuals caught during this study were also larger (with average standard lengths of 220.0 ± 26.3 mm (mean ± SD) for all males and 187.5 ± 22.8 mm for all females caught in this study) than those reported by Tanaka (1964). This means that from 1964 to 2019, the length of reproducing Hasu male and female individuals increased by approximately 37.5% and 44.2%, respectively. The increase in length may be attributed to a combination of factors, including improved fishery management practices, lack of competition for prey resources due to the decline in population size, and habitat restoration efforts that create more favorable conditions for fish growth (Tsunoda 2023). However, there is a need for focused research that incorporates the environmental factors in the Lake Biwa ecosystem to better identify which scenario is more likely. While males were larger in length and weight, females displayed slightly higher gonad weights and gonado-somatic indices, potentially reflecting greater reproductive investment (Gui & Zhou 2010).

Gut content analysis revealed, for the first time ever, the feeding behavior of Hasu fish during the reproductive season in the rivers. Ayu fish dominated the diet of Hasu fish, with limited feeding activity observed at the beginning of the reproductive season (Figure 3.2). Hasu fish seem to allocate their energy resources strategically at the beginning of the reproductive season by temporarily reducing their feeding activity. This is probably to prioritize energy-demanding reproductive activities like courtship, mating, and spawning (McBride et al. 2015).

All individuals caught during reproductive season had a Fulton's condition factor (K) of

greater than 1, indicating that all migrating individuals were healthy individuals (Mozsár et al. 2015, Figure 3.1). The decrease in *K* value during the August catch could be a result of one of two likely scenarios. First, there could be a potential temporary decline in the overall health and condition of the fish population in the river. Several factors may contribute to this decline, including environmental changes, rising water temperatures and potential alterations in food availability (Ficke et al. 2007). The subsequent increase in *K* value in September may indicate a recovery or a response to changing environmental conditions. This, in theory, explains why Hasu fish feed during the reproductive season. Secondly, it is also possible that the decline in *K* value was a result of new individuals approaching the study area. To further assess the likelihood of the second situation, the $\delta^{13}C$ and $\delta^{15}N$ in Hasu muscle and mucus tissues could provide information about the differences in the timing of upstream migration among individuals. The next section discusses these variations.

3.5.2 Multi-tissue stable isotope ratio analysis reveals variation in timing of feeding after upstream migration

Stable isotope ratio analysis of $\delta^{13}C$ and $\delta^{15}N$ revealed a recent change in diet in migrating Hasu individuals (Figure 3.3). In the June sampling, both males and females exhibited higher $\delta^{13}C$ and $\delta^{15}N$ in muscle tissue, with slight fluctuations across the reproductive season, compared to mucus tissue, which had distinct observable changes across the reproductive season. These observed changes indicate a recent change in diet of Hasu fish in Shiotsuo River, and perhaps the migration of Hasu individuals from the Lake Biwa to Shiotsuo River. Generally, Lake Biwa has lower $\delta^{13}C$ and higher $\delta^{15}N$ when compared to its tributaries (Yamada et al. 1998, Maruyama et al. 2001, Ito et al. 2015, Sawada et al. 2019). This is largely due to the differences in phytoplankton and periphyton and their reaction rates as primary producers. Thus, evidence of recent migration can be found in the gradual changes in the faster mucus tissue and the little or no change in the slower muscle tissue. The $\delta^{13}C$ ratios gradually increased in mucus tissue while the $\delta^{15}N$ ratios in mucus gradually decreased (especially after July) across the

reproductive season. The little or no change in muscle $\delta^{13}C$ and $\delta^{15}N$, due to the slow turnover rate, further supports this recent diet switch and perhaps the recent migration of Hasu fish from Lake Biwa to Shiotsuo River.

Furthermore, the characteristic shifts in $\delta^{13}C$ and $\delta^{15}N$ of mucus tissue across the reproductive season suggest time lags and differences in the timing of feeding between males and females, with some males feeding earlier in Shiotsuo River (Figure 3.3). This finding further cements the observations by Imamura (2018) during a study on the shorelines of Lake Biwa. By angling, Imamura (2018) observed that Hasu individuals were caught in an approximate ratio of 3 males per female, likely due to some early migrations in the males. Our stable isotope data also suggests that, even within the same sex group of Hasu fish, there are differences in the onset of feeding (and perhaps timing of upstream migration) in rivers. It is likely that $\delta^{13}C$ and $\delta^{15}N$ in the muscle and mucus tissues are similar between the individuals entering the river at different times (e.g., those in June and those in August). Based on the assumption that all migrating individuals are in equilibrium with their former environment and begin feeding on a consistent diet soon after upstream migration, then sampling at different times should potentially reveal early and late migrators.

In essence, if a Hasu fish caught in August has a similar $\delta^{13}C$ and $\delta^{15}N$ to a Hasu fish caught in June (with $\delta^{13}C$ and $\delta^{15}N$ below the average of the $\delta^{13}C$ and $\delta^{15}N$ in the August catch and is closer to the $\delta^{13}C$ and $\delta^{15}N$ of the lake), then it can be deduced that the Hasu fish caught in August is a late migrator. On the other hand, a Hasu fish caught in August with an above average $\delta^{13}C$ and $\delta^{15}N$, closer to the $\delta^{13}C$ and $\delta^{15}N$ equilibrium of the river, then it can be deduced that the Hasu fish is an early migrator and has been in the river for some time. Early and late migrations have been documented in several species (Kynard & Horgan 2002, Quinn et al. 2007). Hasu fish, particularly the male individuals, are known to exhibit intraspecific aggression, especially toward other males in the same area (Hori 2022). Therefore, having both early and late migrations could represent an evolutionary response to mitigate competition

among migrating Hasu fish.

In conclusion, this study found that the standard lengths of migrating Hasu fish have increased by over 37% since the 1960s despite the continued population decline for the past 70 years. For the first time ever, the study found that Hasu fish exhibit feeding behavior in the river, primarily on Ayu fish, during the reproductive season. This study also revealed that healthy mature individuals approached Shiotsuo River during the reproductive season, with males likely arriving earlier than females. Using $\delta^{13}C$ and $\delta^{15}N$ in muscle and mucus tissues also revealed variation in the onset of migration between individuals and between sexes, with males perhaps slightly migrating earlier than females. These crucial insights are fundamental to the management and conservation of Hasu fish and its ecosystem. As an apex predator and by feeding on important fishery species such as Ayu fish, we conclude that Hasu fish is important for maintaining function and balance in the Lake Biwa ecosystem. Therefore, when setting fish traps in the rivers, the differences in the timing of upstream migration in Hasu fish needs to be carefully considered. We recommend having perennial rivers accessible and free of barriers (e.g., dams) to the fish from June to September, when Hasu reproductive migration is most evident.

4 Using micro-CT derived bone density in the skulls of the vulnerable *Opsariichthys uncirostris uncirostris* (Hasu fish) to explore the health of its migrating population in a Lake Biwa tributary

4.1 Chapter summary

I used micro-CT and inferential statistics to determine whether the relative bone density changes in the skulls of adult Hasu fish reflected the overall health of the migrating Hasu fish population. The relative bone density significantly decreased as standard length and condition factor (*K*) increased in both sexes. This negative relationship is likely due to age and hormonal effects in the fish. Results from the bone density analysis also indicated that male Hasu fish had lower relative bone density than females during peak reproductive migration, i.e., July to August. On average, male Hasu fish are larger in length and weight than females, and in many species, females prefer larger males to smaller males, viewing their size as an indicator of genetic fitness and their ability to provide protection. Resources in the skulls of Hasu males may be distributed in such a way that increases reproductive success, i.e., size at the expense of quality. In addition, individuals with slightly less dense bones, particularly males, appeared later than those with denser bones during the peak of the reproductive season. Hasu fish, especially males, are aggressive, often chasing and biting other males in the same area. The high mechanical demands involved with such aggression, often require resource mobilization from various tissue compartments and could explain the slightly lower density in the latter half of the peak migration. In addition, Hasu individuals that migrated earlier and later during the reproductive season may have more energy reserves than those that had been in the river for some time, hence the variable bone density between individuals. The findings, especially regarding the bone density trends of males, suggest a possible recovery after a stressful period, thus indicating a healthy migrating population of Hasu fish. This study serves as a foundation for future studies on bone density of Hasu fish and other species in various ecosystems.

4.2 Introduction

Understanding bone density variations in Hasu fish, especially between sexes, can provide insights into species-specific adaptations related to reproductive behavior (Martin et al. 2022). In Chapter 3, it was deducted that Hasu fish migrate and initiate feeding at different times. The environment and dietary factors can affect nutrient recruitment in tissues, such as bones, and have an impact on the overall health of the fish (Hodgson et al. 2008).

Fish, such as the small teleost *Danio rerio* (zebrafish), which rely mostly on their skeletal architecture for structural support and mineral homeostasis, have been widely used to model skeletal morphogenesis in human skeletons due to similarities in developmental mechanisms (Javidan & Schilling 2004, Lall & Lewis-McCrea 2007). The assessment of bone density offers a unique perspective in determining the intricacies of fish physiology, and consequently provides insights into the health status of individuals (Lall & Lewis-McCrea 2007). The skeletogenesis of fish is a progressive, often involving mineralization and compositional modulation of skeletal tissues (Hodgson et al. 2008). Such a progressive process reflects the interplay between environmental factors and endogenous physiological processes (Witten & Huysseune 2009). By integrating bone density analysis in the study of the potamodromous fish, this study has potential to unveil a unique dimension linked to the reproductive migration of the species. Potamodromy, characterized by fish that complete their life cycles within freshwater systems, often involves extensive upstream migrations for spawning purposes (Lucas & Baras 2008, Benitez et al. 2015).

The interplay between bone density and reproductive migration holds profound ecological significance. Changes in bone density can offer insights into the energetic demands and physiological adaptations associated with migratory endeavors (Ambrosi et al. 2011). As potamodromous species navigate challenging aquatic environments during reproductive migrations, alterations in bone density may serve as indicators of the metabolic investments required for successful migration and subsequent reproduction (Lall & Lewis-McCrea 2007,

Ambrosi et al. 2011). Understanding this relationship not only enhances our comprehension of the life history strategies of potamodromous fish but also provides a valuable tool for evaluating the impact of anthropogenic activities on critical migratory corridors, ultimately contributing to the conservation and sustainable management of these essential fish populations (Hodgson et al. 2008, Witten & Huysseune 2009). Understanding bone density variations in fish, especially between sexes, can also provide insights into species-specific adaptations related to reproductive behavior (Martin et al. 2022). The environment and dietary factors can affect nutrient recruitment in tissues, such as bones, and have an impact on the overall health of the fish (Hodgson et al. 2008).

Conventional health measurements like Fulton's condition constant (*K*), even though widely used, cannot adequately identify drastic changes in fish energetics especially during the reproductive season when drastic weight changes can be observed due to reproductive behavior (Koops et al. 2004, Mozsár et al. 2015). By assessing the bone density, however, it should be possible to identify sudden changes in the health or energetics of migrating populations (Louis et al. 2022). According to Wolff's Law on bone remodeling, the structure (and consequently density) of bone tissue in healthy individuals will adjust in response to the mechanical forces and stresses applied to it (Frost 1994). The novel technique micro-focus computed tomography (micro-CT), coupled with calcium hydroxyapatite (CaHA) phantoms, provides a powerful tool for studying bone density in fish (Liao et al. 2023). Unlike conventional methods of assessing bone density, e.g., chemical methods that may alter the structure and composition of bones, micro-CT provides high-resolution three-dimensional imaging of bone structures, allowing for detailed quantitative analysis of parameters without altering the structure or composition of the bones (Donnelly 2011, Broeckhoven et al. 2017, Gutiérrez et al. 2018). The inclusion of CaHA phantoms in micro-CT protocols promotes standardization by mimicking the X-ray attenuation properties of bone, therefore enabling calibration of CT images to bone density (Schweizer et al. 2007). This calibration is crucial for accurate and reliable measurements of bone density

(Schweizer et al. 2007, Liao et al. 2023). The non-destructive nature of micro-CT also allows us to examine precious specimens and allows us to make informed decisions on the status of internal structures with minimal damage to the specimen (Jonsson & Jonsson 2014).

Opsariichthys uncirostris uncirostris (Hasu fish), an endemic yet vulnerable potamodromous fish that relies on lake–river migration for its reproductive migration in the Lake Biwa ecosystem, migrates and initiates feeding at different times in Lake Biwa tributaries (Chapter 3). Hasu fish and its related species in Asia, which lack dental teeth, have evolved unique jaws to catch fish within the constraints of the cyprinid family (Okuda et al. 2014). Although the cost of migration has been discussed in evolutionary ecology of migratory fishes, not much is known about the changes in bone density associated with individuals migrating and feeding at different times. Therefore, this research aimed at exploring the health of the vulnerable potamodromous Hasu fish by using micro-CT derived bone density variations in its skulls.

4.3 Materials and Methods

4.3.1 Description of study site

The study was conducted in the lower reaches of the Shiotsuo River, which flows into Lake Biwa from the north. The river has a total length of 9 km and a basin area spanning 21.8 m^2 (Ministry of Environment, Japan 2023). The river flows through mountainous terrain (95.8% of the total river catchment area), contributing to a steep gradient of 9.5 m/km, making it one of Lake Biwa's steepest rivers and a preferred reproductive upstream migration route for Hasu fish. Due to its steep gradient, the river is fast-flowing, and it is also characterized by gravel bottom substrates, all of which have been identified to be key drivers of Hasu reproductive migration (Chapter 2). In addition to the ease of sample collection, Shiotsuo River is perennial, and there are no high weirs in the middle and lower reaches of the river, allowing for natural upstream migration of Hasu fish, as well as Ayu fish. Ayu fish serves as the primary food source for Hasu fish in the Lake Biwa ecosystem (Tsunoda et al. 2015, Chapter 3). These

characteristics make Shiotsuo river a conducive environment for conducting bone density studies on Hasu fish during its reproductive upstream migration.

4.3.2 Fish sampling and biometric measurements

Hasu fish samples were collected monthly in the lower reach (2–3 km from the river mouth) of Shiotsuo River from May to September 2019 using cast nets. The sampling was systematic and a target of 20 Hasu individuals was set for each sampling. Wet weight (g) and standard length (mm) were measured (to the nearest 0.01 g or 0.1 mm) in the field. fish were put in individual Ziplock bags and kept on ice in a cooler box while in the field. After the field sampling, samples were transported to the laboratory at Ryukoku University and kept frozen below –22.5°C until analysis. The Fulton's condition constant (K) was calculated for each fish according to Equation 3.1

4.3.3 Sample preparation and micro-computed tomography

Prior to micro-CT scanning, the samples were placed in 70% ethanol for at least 48 hrs. to facilitate water removal. After the 48 hrs. had elapsed, the samples were cut along the dorsoventral axis just before the pelvic fin. This was done to ensure that the samples fit into a scanning container and field of view of the micro-CT scanner. The samples were then washed in absolute ethanol to remove any impurities on the surface of the fish and fixed in the scanning container together with a MicroCT-HA phantom (QRM, Möhrendorf, Germany) containing 5 rods of Calcium hydroxyapatite (CaHA) with known densities (0, 50, 200, 800, 1200 mg CaHA/cm^3, respectively). The phantom was secured to the sample by means of Sellotape® (Figure 4.1a). All samples were scanned at 70 kV and 40 μA with a 1024 × 1024 resolution and YZ smoothing using an inspeXio SMX-100CT Micro Focus X-Ray CT System (Shimadzu Corporation, Kyoto, Japan). The slice size and voxel size were 0.079 ± 0.009 (mean ± SD) and 0.041 ± 0.043 (mean ± SD), respectively. The scan files were then exported as 8-bit bitmap files (Figure 4.1b) for subsequent processing in 3D slicer (a 3D imaging freeware).

4.3.4 *Measuring relative bone density in skulls of Hasu fish*

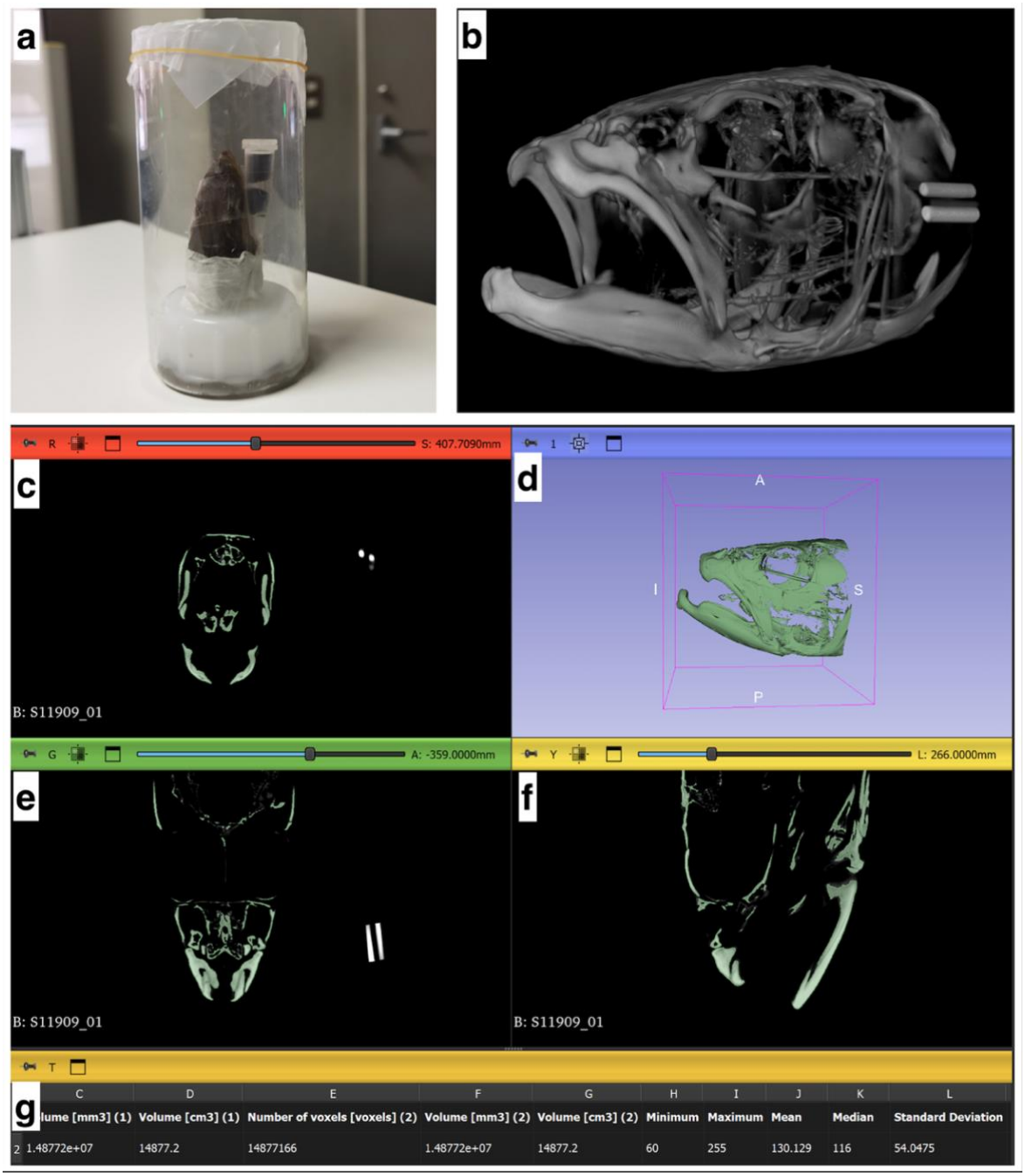

Figure 4.1. Workflow for assessing relative bone density using micro-CT. a: The sample is fixed in scanning container together with CaHA phantoms. b: The scanned images are exported as 8-bit bitmap files for processing in 3D Slicer. c–f: Bones are then segmented using Otsu thresholding (pixel intensity range 60-255) and unwanted regions removed by using the scissors tool within the Multiview in 3D Slicer. g: The pixel intensity in the segmented area superimposed on the original scan is then recorded using the statistics function in 3D Slicer.

Bones in the head scans were segmented (until the preopercle) using Otsu Thresholding (pixel intensity thresholding range: 60-255) due to its simplicity and speed in the Segmentation

editor module of 3D slicer (Figure 4.1c–f, Wallner et al. 2019). The average pixel intensity, i.e., the specific area of the scan that has an accompanying value of x-ray absorption strength (on a grayscale, weak absorption is dark (black or 0 grayscale value) and strong absorption is bright (white or 255 grayscale value); Landis & Keane 2010), for the segmentation on skull bones, superimposed on the original scan, was then recorded using the Statistics function of the Segmentation module of 3D slicer (Figure 4.1g). Similary, the average pixel intensities for each of the 5 phantom rods were obtained and used to create calibration curves (in the slope-intercept form) for converting pixel intensity of the scans to bone density (mg CaHA/cm^3). The R^2 value in all phantom calibrations was 0.99.

4.3.5 Data analysis and interpretation

All data analyses were conducted in R ver. 4.3.1 software. The relationship between the biometrics standard length (mm), sex and condition factor (*K*), as explanatory variables, and relative bone density (as a response variable) were assessed using a generalized linear model (GLM) with a guassian family [base::glm() in R]. Variable selection for the final model was then achieved through forward and reverse stepAIC [base::stepAIC() in R].

The asessment of bone density trends across the sampling period was done in two steps. First, the non-parametric Kruskal-Wallis test [base::kruskal.test() in R] was used to assess whether the observed relative bone density trends, with the interaction between sex and sampling month as an explanatory variable, were significant. If the Kruskal-Wallis test was significant, then a multiple comparison test using the Steel-Dwass test was conducted using the NSM3::pSDCFlig() with "Monte Carlo" as a method in the function (due to the relatively small sample size in this study). The advantage of using the Steel-Dwass test over conventional methods, such as the Dunnet method, is in its ability to solve multiple comparison problems more easily (Takagi et al. 2003). When statistically comparing the relative bone density between males and females of Hasu fish, only datasets from the July catch (n = 10 males, n = 10 females) and August catch (n = 9 males, n = 11 females) were used due to sample size limitations. The

sample sizes in the June catch (n = 11 males, n = 1 female) and September catch (n = 3 males, n = 2 females) were not large enough to perform statistical comparisons. However, the analysis was repeated for male individuals only comparing the June catch (n = 11 males), July catch (n = 10 males), and August catch (n = 9 males) due to a sufficiently large enough sample size for statistical analysis.

4.4 Results

4.4.1 Relationship between biometrics and micro-CT obtained bone density

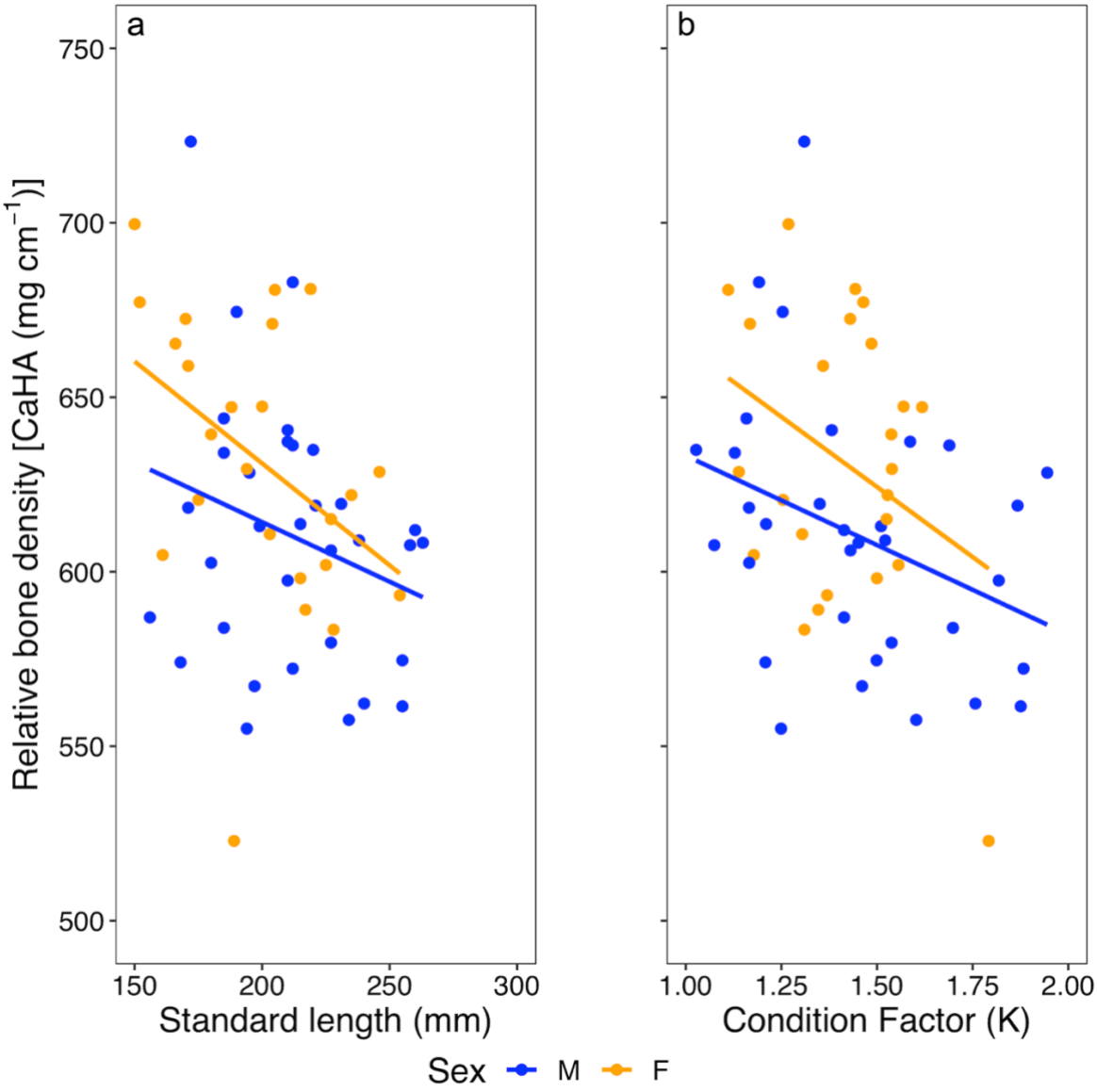

Figure 4.2. Relationship between biometrics, standard length (a) and Condition Factor (b), with relative bone density. The blue and orange points indicate male and female Hasu fish, respectively. The solid lines indicate the linear models for the relationships.

In both males and female Hasu fish, bone density decreased with increasing standard length and condition factor (*K*) (Figure 4.2a–b). The GLM model with gaussian family and stepAIC revealed that there was a negative relationship between relative bone density and the biometrics standard length, condition factor and sex (Table 4.1). However, only the standard length and condition factor (K) had a significant negative effect on the relative bone density in the GLM ($p < 0.05$). This was contrary to the expectation that healthier individuals would have a larger bone density than slightly less healthier individuals.

Table 4.1. Coefficients (estimates ± standard errors) on relative bone density evaluated in relation to biometrics (standard length, condition factor and sex) as explanatory variables using general linear models (GLMs) with a gaussian family selected via stepAIC.

	Coefficients (estimates ± standard errors)		
(Intercept)	778.56	±	42.20***
Standard length (mm)	–0.38	±	0.17*
Condition factor (*K*)	–50.23	±	21.63*
Sex	–14.47	±	9.88

Significance levels (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$)

4.4.2 Bone density distribution across the reproductive season

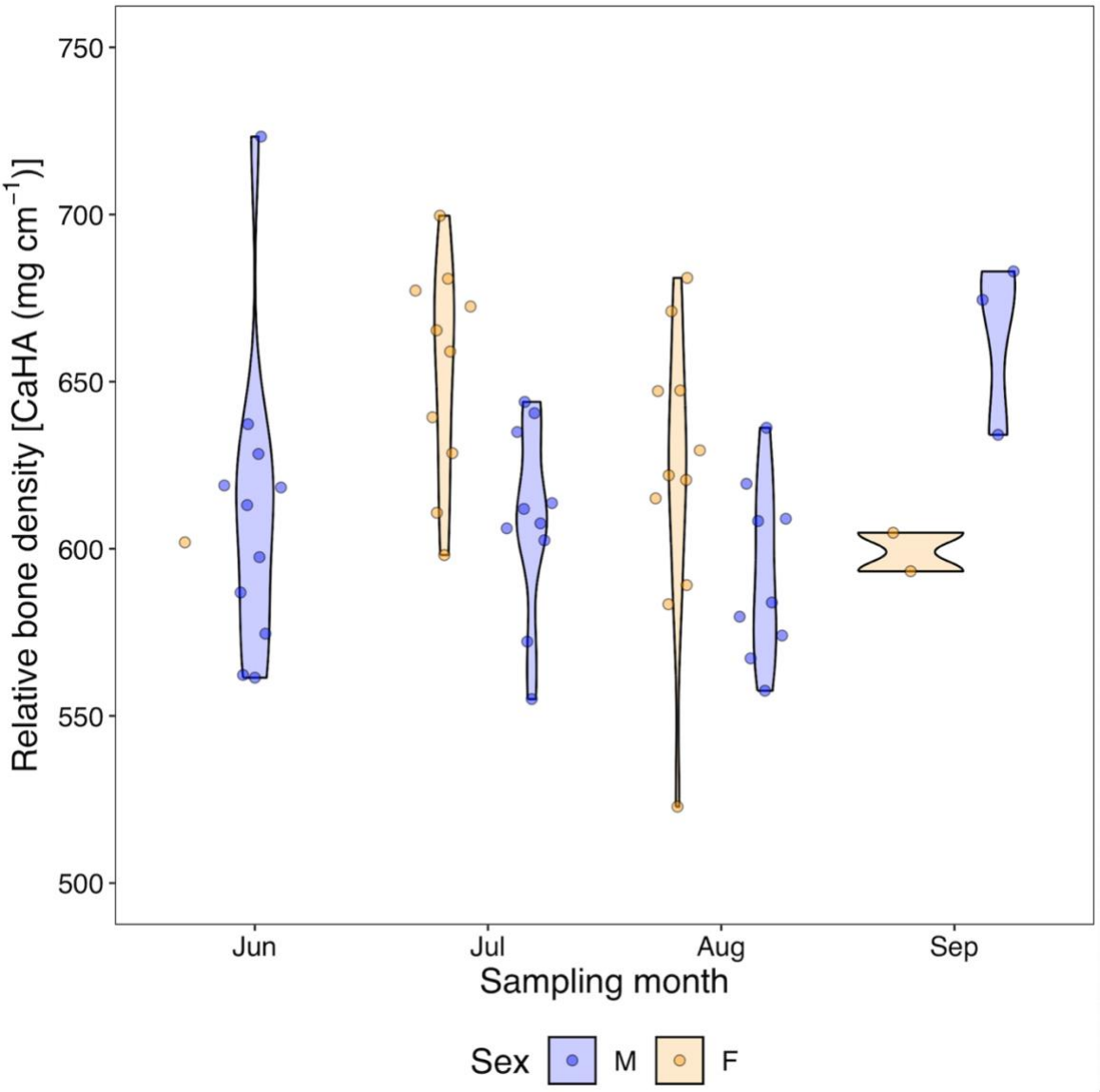

Figure 4.3. Relative bone density [CaHA (mg cm^{-1})] changes in Hasu fish caught across the reproductive season. The blue and orange violin plots (with points) indicate male and female Hasu fish, respectively. The violin plots indicate the distribution of relative bone density, where the width of the plot at any given point indicates density of the data. Violin plots and points were jittered to align with each other. In May, no fish were caught despite sampling efforts; as a result, it was excluded from the plots.

There was a significant difference in bone density between the July and August catches (Kruskal-Wallis chi-squared = 12.34, *d.f.* = 3, $p < 0.05$). Bone density was lower in the August catch when compared to the July catch for both males and females (Figure 4.3). Although bone density was higher in females than in males between the July and August catches, the comparisson test revelead that the differences were only significant between females caught in July and males caught in August (Figure 4.3, W statistic = 4.39, $p < 0.05$). In males only, the bone

density was lower for each sampling month when compared to its previous month except in September when the bone density was higher than the previous month.However, the Kruskal-Wallis test revealed that the observed differences in relative bone density were not significantly different from each other. These results indicate that females have higher densities when compared to males during the reproductive season.

4.5 Discussion

4.5.1 Effect of age and environment on bone density

In this study, the relative bone density decreased as standard length and condition factor increased in both sexes. Thus, it could be deducted that slightly larger Hasu fish, with less dense skulls, were migrating later than the slightly smaller Hasu fish (Figure 4.3, Table 4.1). As species grow, the rate at which materials, especially calcium hydroxyapatite, replenish in bones decreases probably due to age and hormonal effects in the fish (Suarez-Bregua et al. 2018, Liao et al. 2023). However, in the case of Hasu fish, it is highly likely that the observed decline is due to hormonal effects, since migrating individuals during the reproductive season are fall within the same older age group (Tanaka 1964, Tanaka 1970, Tsunoda 2023). Fish bones play an important role in resource mobilization by acting as reserves for important nutrients. These nutrients, e.g., calcium hydroxyapatite, lipids, and proteins, are not only important for structural integrity of the skeletal system but may also act as an energy source for the fish in a stressed environment (Lall & Lewis-McCrea 2007, Liao et al. 2023). Larger individuals require more energy to move their large bodies (Kendeigh 1970). This would explain the slightly lower bone density in larger individuals. In addition, one would expect healthier individuals to exhibit higher densities compared to less healthier individuals. Even though all migrating individuals were healthy ($K > 1$), to the negative relationship between condition factor (K) and bone density suggests some sort of resource investment strategy by Hasu fish during their reproductive migration, perhaps to other tissue such as muscle and gonads which play a more direct role in spawning (Eliassen & Vahl 1982, Memis & Gün 2004). Further research is required to ascertain

resource utilization strategies from various tissues in Hasu fish.

4.5.2 Sex roles influence bone density in Hasu fish

Results from the bone density analysis also indicated that male Hasu fish had lower relative bone density than females (Figure 4.2, Figure 4.3). Besides the hormonal differences between males and females, male Hasu fish are, on average, larger in length and weight than females (Tanaka 1964, Chapter 3). The effect of size and its role in reproduction might help explain the lower bone density in males than in females. A larger head could be a consequence of evolutionary processes that make males appear more threatening to other species, thus appealing to females during reproductive migration. Generally, females in many species prefer larger males, viewing their size as an indicator of their ability to provide protection (Zahavi 1975, Lindström 1988, Sih & Bell 2008). In addition, large sizes may be an indicator of good health and genetic fitness in individuals (McGinleay et al. 2011). Therefore, resources in the skulls of Hasu males may be distributed in such a way that increases reproductive success, i.e., size at the expense of quality. In addition, individuals with slightly less dense bones, particularly males, appeared later than those with denser bones during the peak of the reproductive season (around July-August). Hasu fish, especially males, are known to be aggressive, often chasing and biting other males in the same area Hori (2021). Having denser bones during the peak of the reproductive season (around July-August), when more females are migrating, could provide a better reproductive advantage, not only as a weapon but also during resource mobilization.

The higher bone density in June and September when fewer individuals are migration could be as a result of one of three likely scenarios: lack of competition between individuals, early and late migrations within the Hasu fish population, or a response to strenuous reproductive activities (Figure 4.3). First, it is possible that due to the reduced numbers, there is reduced competition for mates and consequently the absence of high energy demanding activities like chasing other individuals (Kaushik & Medale 1994, Calow 1995). Alternatively, it is possible that the higher bone density in June and September is because of new individuals

approaching the reproductive migration sites. There are possible differences in the onset of feeding and consequently upstream migration between individuals (Chapter 3 It is likely that these individuals, during the early and late migration waves, have enough energy reserves to undertake their reproductive migration journeys and this is not reflected in the changes in bone density. Finally, the higher bone density could be due to bone remodeling after strenuous reproductive activity (e.g., the upstream swim and spawning). According to Wolff's Law on bone remodeling, the structure (and consequently density) of bone tissue in healthy individuals will adjust in response to the mechanical forces and stresses applied to it (Frost 1994). Bone remodeling is a complex process that involves the conversion of mechanical signals into biochemical signals in cellular signaling (Robling & Turner 2009). There is evidence of mechanically induced bone remodeling in the jaws of teleost fish, such as cichlids (Gunter & Meyer 2014). As such, the mechanical demands of reproductive activities could induce remodeling response in the skulls of Hasu fish to cope with this stress. However, there is a need for further investigations on the behaviors of Hasu fish before and after the peak migration period to draw conclusions on which scenario is more likely. For example, future studies may consider exploring the immediate impact of various stimuli (e.g., mechanical and chemical stimuli) on bone density in Hasu fish. This would not only provide insights into the dynamic nature of Hasu fish bone remodeling in response to varying environments but could also inspire the development of new biotechnological approaches for bone tissue engineering and regeneration in humans, as is the case with zebrafish and other marine species [Javidan & Schilling 2004, Lalzawmliana et al. 2019].

In conclusion, this study demonstrated variations in bone densities between male and female Hasu fish, with females having higher bone density than males. I recognize the limitations in this study and that the results must be treated with caution due to the relatively low sample size. However, by using inferential statistics, this study was able to deduce that not only was the Hasu population healthy, but the distinct changes in bone density during each catch

are likely due to resource mobilization strategies in Hasu fish to ensure successful reproduction.The findings from this study serve as a foundation for ecologists and biomechanics hoping to study bone density changes in Hasu fish and other species in various ecosystems.

5 General Discussion

5.1 Chapter summary

In this chapter, I summarize and discuss the significance of this doctoral dissertation in Hasu migration ecology and environmental studies.

5.2 Contribution of this thesis to Hasu reproductive migration

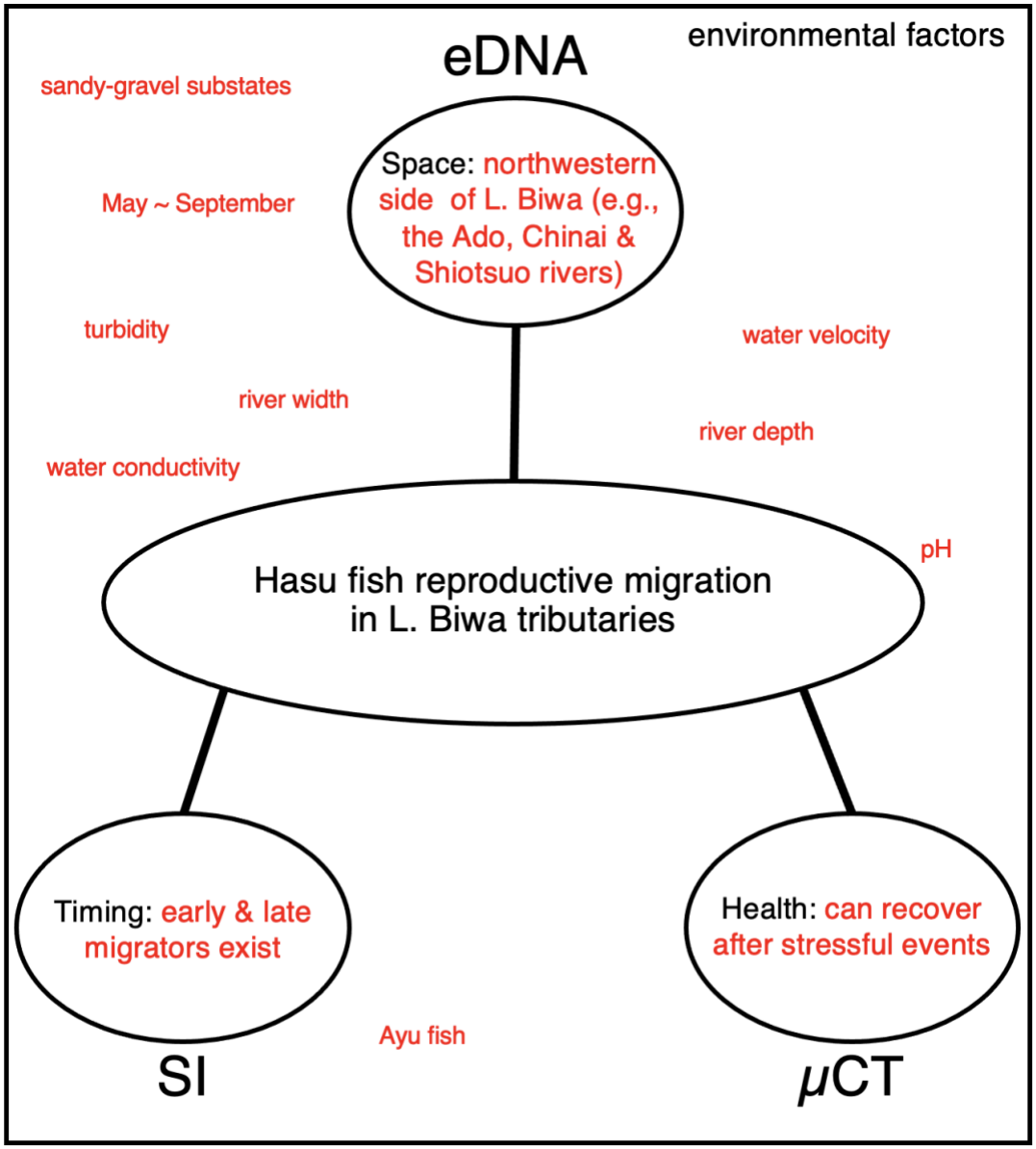

Figure 5.1. A summary of the main findings in this study (in red) obtained using the three integrated migration ecology techniques (i.e., eDNA, stable isotopes and micro-CT). In summary, Hasu fish migrates mostly in the northwestern tributaries of L. Biwa. There is also evidence of early and late migrations within the population. The population is also able to recover after stressful events. Environmental factors can affect the migration of Hasu fish to the tributaries.

The main contributions of this study are summerized in (Figure 5.1).Using data from 32 rivers covering the known range of Hasu fish around Lake Biwa tributaries, the study identified migration hotspots for the vulnerable species within the Lake Biwa ecosystem. Hasu fish were observed to predominantly migrate to the northwestern side of Lake Biwa, where perennial rivers such as the Ado, Chinai, and Shiotsuo, with sandy gravel substrates are located. In addition, fast flowing water at the river mouth also contributes significantly to the presence of Hasu in rivers and consequently river choice. This reinforces Imamura's observations during a 2018 study along the shoreline of Lake Biwa, where more Hasu fish were observed in the vicinity of open watercourses and less near dried watercourses. However, it seems once Hasu has identified the rivers, the flow within the channel is irrelevant. Similar to Maruyama et al. in 2018, I found that Hasu fish reproductive migration typically took place from May to September, with the peak migration observed between July and August in most of the rivers. Unlike Maruyama et al., however, I observed a slight increase in Hasu presence from August to September in some rivers (e.g., in the Shiotsuo, Ishida, Ado and Kamo rivers; Figure 2.2) providing evidence of late migrations in some individuals. Hasu fish, which are at least 37% larger in length than those reported by Tanaka in the 1960s, were observed to migrate and begin feeding, particularly on Ayu fish, at varying times in the rivers. To the best of my knowledge, this is the first account of Hasu fish feeding behavior during its reproductive migration in Lake Biwa tributaries.

Differences in migration timings between male and female species in the Lake Biwa ecosystem are not uncommon. A recent study by Takenaka et al. on *Gymnogobius isaza* in 2023 revealed that male spawning migrations preceded those of females. In this study, I further demonstrated that the differences in migration were not only between males and females but also within males only and females only. These differences were more pronounced in male Hasu than in female Hasu, likely representing an evolutionary response to reduce competition between individuals. These sex-related differences were also apparent in the skulls of Hasu fish,

with males having less dense skulls than females. Additionally, individuals with less dense skulls (usually larger individuals) migrated later than those with denser bones (slightly smaller individuals). However, since migrating Hasu fish typically fall under the same age group (2–3-year-old), it is likely that body size was not a significant factor on the bone density. Further investigation on the effect of body size (e.g. standard length), age and sex on Hasu fish bone density is required. Based on my preliminary survey of Hasu fish at the Lake Biwa Museum in 2020 and observations by Hori et al. in 2021 during a laterality study on Hasu and Ayu fish, it is evident that the species is aggressive, often bumping headfirst into obstacles along its path. There is evidence of traumatic injuries and deformities in fish due to aggression and accidental collisions (Noble et al. 2012). However, to the best of my knowledge, there is no information on such trauma and injuries in Hasu fish. It is important to recognize that some Hasu individuals may have weaker skulls and may encounter obstacles along their path. Evidence derived from the micro-CT bone density indicate possible recovery from stress. Therefore, there is need for detailed studies assessing the effects of stressors (e.g., mechanical, and chemical), aggressive behavior, possible trauma, and variation in skull density on the survival or reproductive success of migrating Hasu individuals. After conducting such a study, fisheries experts can only then account for the necessary materials when setting up fish traps, creating weirs, or modifying riverbanks.

It is also paramount that the migration corridors of Hasu fish be accessible to prevent the fish from expending unnecessary energy while trying to overcome obstacle during its reproductive migration. Based on the findings from this study, it is also recommended to keep rivers on the northwestern side of Lake Biwa, such as Ado, Shiotsuo, and Chinai Rivers, free of migration barriers between May and September to ensure the successful reproductive migration of Hasu fish. Evidence from this study also suggest that Hasu fish approach the river mouth of many rivers on all sides of Lake Biwa. Another potential approach to recover the population of Hasu fish is to modify some smaller rivers to include fast-flowing water and have

gravel and sandy bottom substrates. This could provide suitable breeding grounds for Hasu fish and potentially increase the population of Hasu fish in Lake Biwa. For the effective conservation and fisheries management of the potamodromous apex predator, yet vulnerable, Hasu fish, the findings from this study should be considered.

5.3 Using integrated approaches has the potential for use in different ecosystems

The integration of eDNA, stable isotope analysis, and micro-CT is a groundbreaking approach for comprehensively studying fish reproductive migration (Figure 1.1). eDNA serves as a powerful tool for monitoring spatial distribution, enabling us to track the presence of migratory fish at various locations (Rees et al. 2014, Iwai et al. 2018). In addition, stable isotope analysis, by deciphering migration history through trophic ecology, unveils the dietary sources supporting reproductive migration (Maruyama et al. 2016, Winter et al. 2019). Simultaneously, micro-CT's non-invasiveness allows for detailed exploration of internal structures, providing insights into resource mobilization strategies during reproductive migration (Broeckhoven et al. 2017). The complementary use of these methods offers a multi-faceted understanding of fish reproductive migration, clarifying not only the physiological and behavioral aspects but also providing critical information on habitat utilization, resource dynamics, and spatial distribution (Lucas & Baras 2008, Turbek et al. 2018). This integrated approach is fundamental for informed conservation and management strategies, addressing the diverse challenges associated with the preservation of migratory fish populations and fostering the sustainable management of aquatic ecosystems, as follows:

First, the integrated approach can be used to monitor invasive species. For example, this holistic approach can be used to study the invasive Hasu fish in Kyushu. Although the Hasu fish is considered vulnerable in the Lake Biwa ecosystem, it is invasive in the irrigation ditches of northern Kyushu region (Kurita et al. 2008, Kurita et al. 2014). In this region, Hasu fish thrives despite low food availability (Kurita & Onikura 2016). By integrating eDNA to study the spatial distribution of Hasu in its non-native regions, along with stable isotopes that explore

the species' trophic behavior, we can better understand why the species has been thriving in its non-native Kyushu. In addition, by using micro-CT to compare the internal anatomies of native populations in Lake Biwa and those of invasive populations in Kyushu, we can potentially expose differences between the two populations and reveal otherwise obscure details. These obscure details, like differences in bone morphometrics, can be used to make informed conservation decisions and perhaps help recover the declining population of Hasu fish in its native environment (Thurrow 2016). The applicability of this integrated approach is not limited to Hasu fish and the Lake Biwa ecosystem. In fact, this integrated approach has the potential to be applied to other ecosystems and vulnerable species.

Secondly, this integrated approach can be used to study other vulnerable species in different ecosystems. For example, in the Lake Malawi ecosystem. Like Lake Biwa, Lake Malawi is one of the ancient lakes globally and is home to diverse endemic species (Turner et al. 2019). One species currently encountering challenges in the Lake Malawi ecosystem is *Opsaridium microlepis* (locally known as Mpasa fish), a member of the Cyprinidae family that relies on lake-to-river migration for reproduction, like Hasu fish (Limuwa et al. 2012, Changadeya et al. 2013, Sungani et al. 2016). The species is under threat from overfishing and poisoning by local fishing communities, rendering it endangered (Sungani et al. 2016). The use of an eDNA-stable-isotope-micro-CT integrated approach can help identify migration hotspots, reveal the timings of upstream migration, and determine the health status of the migrating fish. Such information is crucial and could be used to formulate informed conservation and management strategies to help recover the fish population.

Lastly, the integrated approach can be used to assess the effectiveness of conservation efforts. Apart from Mpasa fish, the integrated approach could be used to study the numerous cichlids in Lake Malawi. In recent years, local populations, with the assistance of non-governmental organizations, have aimed to conserve and protect the Lake Malawi ecosystem (Chafota 2005). For instance, community-led sanctuaries have been established to conserve

these important species (Davis 2003, Russell et al. 2008, Jamu et al. 2023). However, there is a need for a scientific-based approach to evaluate the effectiveness of these sanctuaries (Jamu et al. 2011). By using an integrated approach, it is possible to provide a holistic picture of the needs of sanctuaries and their effectiveness. For example, eDNA could be used to monitor the presence of fish in the sanctuaries through periodic sampling, with help from the local communities due to its relative ease of sample collection (Maruyama et al. 2018, Yamanaka et al. 2018). Simultaneously, stable isotope analysis can be used to trace the migratory history of the species and potentially identify other areas requiring conservation attention (Shigeta et al. 2017). Micro-CT imaging can then complement the other two approaches by assessing resource mobilization strategies and shedding light on the adaptability of the cichlids to changing environmental conditions in Lake Malawi (Jamu et al. 2011, Thurrow 2016). This comprehensive approach, integrating distribution monitoring, migratory history, and physiological assessments, could not only enhance our understanding of Lake Malawi cichlid ecology but also inform targeted conservation and management strategies for these unique fish populations.

In conclusion, the integration of eDNA, stable isotope analysis and micro-CT represents a transformative approach to studying fish reproductive migration. Each method contributes unique and complementary information, collectively providing a more comprehensive understanding of the complexities involved in the reproductive migration of vulnerable fish. The combination of non-invasive imaging, genetic monitoring, and ecological tracers not only enhances our ability to decipher the physiological and behavioral aspects of reproductive migration but also sheds light on the broader ecological context and encompasses habitat utilization. By adopting these integrated methods, researchers and conservation practitioners can generate a more detailed and holistic picture of fish reproductive migration, essential for designing effective management strategies and safeguarding the delicate balance of aquatic ecosystems.

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List of Peer-reviewed papers

In this thesis

1. Mvula A, Tawara D, Maruyama A (2024) Using micro-CT to explore bone density variations in the skulls of the vulnerable *Opsariichthys uncirostris uncirostris* (Hasu fish) during reproductive migration to a Lake Biwa tributary. *Plos One*. doi: 10.1371/journal.pone.0310461
2. Mvula A, Maruyama A (2024) An assessment of the potamodromous fish *Opsariichthys uncirostris* (Hasu fish) reproductive migration using stable isotope ratios and biometric data. *Journal of Ichthyological Research.* doi: 10.1007/s10228-024-00965-1
3. Mvula A, Sawada H, Yamanaka H, Maruyama A (2023) Identifying migration hotspots of the potamodromous fish *Opsariichthys uncirostris* in Lake Biwa tributaries using environmental DNA and visual counts during its reproductive season. *Ecological Research*. doi: 10.1111/1440-1703.12433

Not in this thesis

1. Takeuchi Y, Hata K, Sasaki M, Mvula A, Rusuwa B, Maruyama A (2024) Preying on cyprinid snout warts (pearl organs) as a novel and peculiar habit in the Lake Malawi cichlid Docimodus evelynae. *Scientific Reports*. doi: 10.1038/s41598-024-69755-z)

List of Conferences

Poster presentations

1. Mvula A, Tawara D, Maruyama A (March 2023) Using micro-CT to investigate variation in fish jaws. ESJ70. Poster No.: P1-312
2. Mvula A, Maruyama A (March 2022) Exploring the use of micro-computed tomography (μCT) as a tool for studying phenotypic variation in fish populations. ESJ69. Poster No.: P1-339
3. Mvula A, Sawada H, Imamura A, Yuma M, Yamanaka H, Maruyama A (March 2020)

Migratory ecology of Hasu fish to Lake Biwa tributaries using eDNA and isotopic clock analysis. ESJ67. Poster No.: P1-PC-369

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ORIGINAL ARTICLE

ECOLOGICAL RESEARCH WILEY

Identifying migration hotspots of the potamodromous fish *Opsariichthys uncirostris* in Lake Biwa tributaries using environmental DNA and visual counts during its reproductive season

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Abstract

Migration is vital for the continuation of a species. In this study, we explored migration hotspots of the vulnerable Hasu fish in Lake Biwa tributaries using two complementary methods: environmental DNA (eDNA) and visual counts. The study encompassed the known range of Hasu around Lake Biwa tributaries during its reproductive season. Monthly water sampling and visual inspection was conducted, from May to September, in 32 Class A tributaries—at the river mouth and within the river channel. Hasu eDNA was extracted from water samples and quantified using real-time polymerase chain reaction. Environmental factors were also assessed on-site, and their effects on eDNA and visual count trends evaluated using linear models and Akaike information criterion. eDNA was detected at sites where the fish were both observed and not observed. A zero-hurdle model revealed positive correlation between eDNA copies and visual counts of migrating Hasu, with pH having a reducing effect on the relationship ($p < 0.05$). Analysis of Hasu eDNA copies and visual count trends, with environmental factors as explanatory variables, indicates that Hasu is likely to be found in rivers that are wide and deep enough to accommodate migrating individuals, have fast-flowing currents, and sandy-gravel substrates during reproductive migration. Such rivers are mostly located on the western side of the northern basin and include the Ado, Chinai, and Shiotsuo Rivers. These could be considered as Hasu migration hotspots and require protecting if the population of Hasu in Lake Biwa is to be recovered.

KEYWORDS

fish abundance, pH, real-time polymerase chain reaction, species specific quantification, upstream migration

1 | INTRODUCTION

Studies on fish migration have revealed that fish migrate for various reasons, including establishing new feeding grounds, escaping unfavorable environmental conditions, and spawning—all of which are vital for the continued existence of a species (Fryxell, 1991; Holland et al., 2006; Hansson & Hylander, 2009; Thurrow, 2016). However, despite tremendous advances in migration studies, considerable challenges remain when applying conventional

methods to rare and elusive species. For instance, potamodromous fishes, which migrate in entirely freshwater systems, have not received sufficient attention due to difficulties in collecting information (Lucas & Baras, 2008; Thurrow, 2016). These difficulties include challenges like visual counts, which are not easily obtained when the population size is extremely low, and Sr/Ca ratio, which are not applicable to potamodromous fishes.

In recent years, novel techniques, such as environmental DNA (eDNA) analysis, have gained traction in migration studies. eDNA can be extracted from environmental samples, such as sediment and water (Ficetola et al., 2008). The application of eDNA to migration studies is relatively new and is based on the principle that the environment can preserve the molecular imprint of inhabiting species (Ficetola et al., 2008). According to this principle, it is possible to detect organisms without direct observation by using extracted DNA from an environmental sample and conducting species-specific polymerase chain reaction (PCR) detection or quantification. The results from eDNA analysis have helped to identify recently migrating organisms in freshwater systems and, consequently, migration hotspots, defined in this paper as areas with high eDNA copies and fish counts during the reproductive season. This is particularly applicable when sampling is done in surface water, where eDNA can persist for up to a week even after the migrating organism has left the environment, as opposed to sediments, where eDNA has been detected up to 3 months after the migrants have left the environment (Maruyama et al., 2014; Turner et al., 2015). Periodic sampling and monitoring of eDNA copies can also help determine seasonal migration (Laramie et al., 2015; Wacker et al., 2019). Hypothetically, eDNA copies will increase with the arrival of new individuals, remain at a dynamic equilibrium when abundance is sustained, and decrease with the departure of individuals. In addition, eDNA should be absent with the absence of the individuals.

While the application of eDNA in freshwater systems appears to be established, the method faces significant obstacles. One such obstacle in current eDNA studies is its reliability in estimating abundance (or biomass) in migrating populations based on eDNA samples (Harper et al., 2019; Maruyama et al., 2018; Rees et al., 2014; Takahara et al., 2012). Accurate abundance (or biomass) estimates are crucial for making decisions regarding the management and conservation of rare and endangered species. The reliability of this method has been a subject of debate in lotic systems. Iwai et al. (2019) observed that stream flow prevented the even distribution of eDNA, making it difficult to obtain eDNA copies that accurately reflected the number of individuals present in the environment. However, Maruyama et al. (2018) found that stream flow did not have a significant effect on eDNA quantification in the environment. Nevertheless, taking into consideration environmental factors such as pH and temperature, as well as incorporating other methods like visual counting, can improve the efficiency of eDNA analysis as a tool for studying migrating rare and endangered species.

Opsariichthys uncirostris (local name: Hasu) is a potamodromous species that has not been extensively studied since the 1960s. The last comprehensive study on the species was conducted by Tanaka in 1964. Hasu relies on lake-to-river migration for reproduction, and it is the only piscivorous cyprinid fish in Japan, endemic as a subspecies to Lakes Biwa and Mikata (Tabata et al., 2016). Unfortunately, its population has been steadily declining for the past 70 years (Figure 1). Consequently, Hasu is considered vulnerable in Lake Biwa and extinct in Lake Mikata (Ministry of the Environment, Japan, 2020). Hasu spawning is reported to occur in summer, from late May to early August in Lake Biwa and its tributaries (Miura, 1966; Tanaka, 1964). Spawning takes place over sandy-gravel bottoms within inlet streams and the shores of Lake Biwa, primarily involving mature males and females of ages 3 years (average body length: 160 mm) and 2 years (average body length: 130 mm), respectively. Using eDNA analysis, Maruyama et al. (2018) deduced that during the reproductive season in the Chinai River, located on the north-western side of Lake Biwa, the abundance of Hasu gradually increases from May to July and decreases in August. Maruyama et al. (2018) also argued that Hasu inhabits spawning sites for longer times

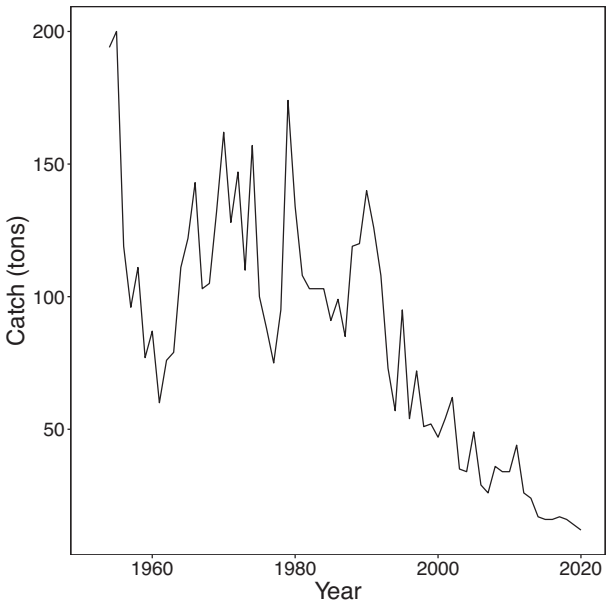

FIGURE 1 Declining trend of Hasu catches in Lake Biwa from 1954 to 2020.

than previously reported. However, Maruyama et al.'s (2018) study was conducted in only one of 117 Class A tributaries (rivers under the control of the National Government) of Lake Biwa and may not provide a complete picture on the range of Hasu migrations in the Lake Biwa ecosystem. A comprehensive study covering a wider range could help identify areas of conservation importance and shed light on why the Hasu population has continued to decline for over 70 years.

Therefore, this study aimed at identifying key rivers (migration hotspots) of the vulnerable potamodromous Hasu in Lake Biwa tributaries by using visual counts and eDNA analysis as complementary methods within a single study. Water samples were periodically collected for eDNA analysis, and Hasu counts (by visual inspection) were concurrently obtained in 32 of the 117 Class A tributaries of Lake Biwa. The study encompassed the range of the species in tributaries on all sides of Lake Biwa. We then used environmental factors to evaluate the trends in Hasu counts and eDNA copy numbers within the 32 tributaries, with the aim of uncovering what parameters made the migration hotspots conducive for Hasu migration.

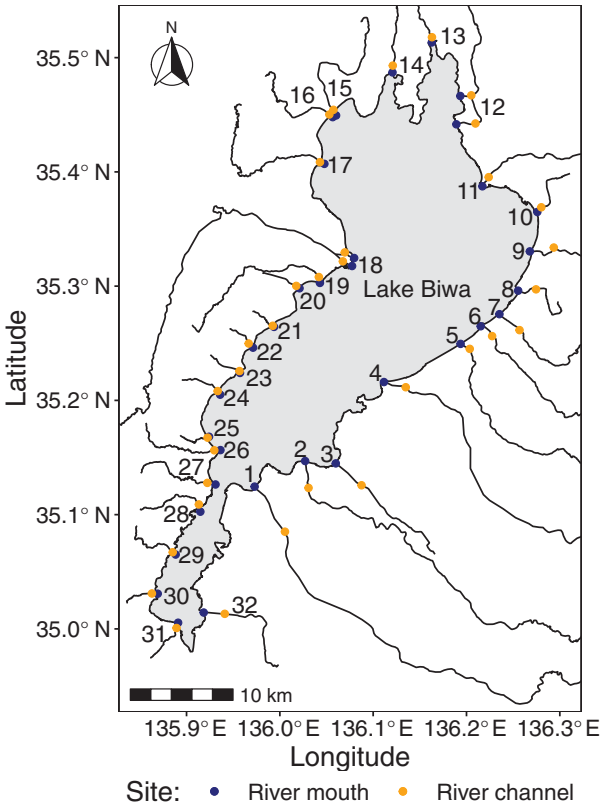

FIGURE 2 Lake Biwa tributaries investigated for Hasu migration in this study. The numbers 1–32 indicate: Yasu (1), Hino (2), Shiratori (3), Echi (4), Uso (5), Inukami (6), Seri (7), Yagura (8), Amano (9), Nagahamashinsen (10), Ane (11), Yogo* (12), Shiotsuo (13), Oura (14), Chinai (15), Momose (16), Ishida (17), Ado* (18), Kamo (19), Wadauchi (20), U (21), Taki (22), Hira (23), Otani (24), Kisen (25), Wani (26), Mano (27), Tenjin (28), Omiya (29), Yana (30), Sagami (31), and Kusatsu (32) Rivers, respectively. Asterisk indicates that the river has two arms feeding into Lake Biwa. Both arms were independently assessed in the study.

2 | MATERIALS AND METHODS

2.1 | Field sampling

Water samples for eDNA analysis were collected once a month, from early May to early September 2019, in 32 preselected rivers out of 117 Class A rivers feeding into Lake Biwa (latitude: 35.00° N to 35.52° N; longitude: 135.86° E to 136.29° E)—selection of the rivers was done based on previous sightings of the fish as well as to encompass all sides of Lake Biwa. Water samples were collected during the daytime within a uniform time range (e.g., 9 a.m. to 10 a.m.), but the time ranges varied among the different rivers. When sampling was not possible due to bad weather, sampling was rescheduled for the next available date. Water samples were collected at the river mouth and within the river channel close to the river mouth (Figure 2). Sampling stations within the river channel were selected based on the presence of a bridge (for easy visual inspection) and absence of backflow. During each sampling, 1 L of water was collected using a polyethylene cup and filtered on site using a glass fiber filter (Whatman GF/F, 0.7 μm nominal pore size, GE Healthcare, Chicago, USA) and a polypropylene filter holder (FH-PP47, ASONE, Osaka, Japan) immediately after sampling to reduce eDNA decay—none of the equipment was reused during the course of a sampling day to avoid inter-sample contamination; the polyethylene cups were one-time-use and appropriately discarded while the filter holders were bleached with 10% bleach solution before reuse on another sampling day (Yamanaka et al., 2018). Each water sample was handled with a new pair of disposable gloves. During filtration, another new pair of disposable gloves was used to avoid sample contamination. Remaining water in the glass fiber filter was then replaced with 99.9% ethanol by filtering approximately 2 mL of ethanol (enough to cover the entire surface of the GF/F on the filter holder) to preserve eDNA (Minamoto et al., 2016), folded in half using forceps, and wrapped in aluminum foil to avoid post sampling contamination and light. During each sampling day, no decontamination by bleaching was done in the field, instead, a new pair of forceps was used for each sample. The wrapped filters were put in separate plastic bags to avoid inter-sample contamination and kept below −20°C using icepacks in a cooler box during

transportation to the laboratory. At the end of each sampling day, 1 L of distilled water was filtered as a negative control and treated in the same manner as the samples. At the laboratory, the filters were kept below −20°C in a freezer until eDNA extraction.

2.2 | Measurement of environmental factors and visual counts

Water temperature (to the nearest 0.01°C), pH, and electrical conductivity (to the nearest 0.01 μS/cm) were measured and collected on site during each sampling using the Hanna HI98130 combo meter (Hanna Instruments Inc., USA); turbidity (NTU) using the Eutech TN-100 turbidity meter (Thermo Scientific, USA); type of substrate (i.e., clay: <0.002 mm, sand: 0.002–2 mm or gravel: >2 mm; Xu, 2004; a binary system was used to indicate presence or absence of sand (S), gravel (G), and clay (C). In this case, if a substrate was sandy-gravel, it was denoted as S:1, G:1, C:0) and river velocity (to the nearest 0.01 cm/s) using the CR-11 current meter (Cosmo Riken Ltd., Osaka, Japan, Lower limit of detection: 4 cm/s); and depth (to the nearest 0.1 m), width (to the nearest 0.1 m), and coordinates (latitude and longitude) using the Huawei P20 lite (Huawei Technologies, Co., Ltd., Shenzhen, China) with the Google Maps application (Google Inc., Cal., USA). Except for the type of substrate (which used the binary system) and coordinates, all environmental factors were collected in triplicates and the averages were used for subsequent analyses. These were selected based on their effect on the amount of eDNA in water bodies and Hasu spawning (Barnes et al., 2014; Maruyama et al., 2018; Miura, 1966). Visual inspection was only done within the river channel. It was not done at the river mouth due to difficulties in collecting information (e.g., absence of bridges, steep banks, and dense vegetation). Visual inspection was done by counting the number of Hasu individuals within a 40-m stretch of each eDNA sampling station—Hasu individuals migrating upstream are large enough (14–25 cm standard length) to be distinguished from other fish from either the bridge or riverbanks. Individual density per unit area was not assessed in this study. Unlike density models, models based on individual counts can accommodate a wide range of predictor variables and are better suited at handling zero-inflated data as well as overdispersion (Dalrymple et al., 2003). Overdispersion and zero-inflation are among the challenges faced when statistically analyzing eDNA copies versus individual count data (Maruyama et al., 2018).

2.3 | eDNA extraction and quantification

Extraction, amplification and quantification of eDNA in the water samples was done as outlined by Yamanaka et al. (2018) and Maruyama et al. (2018) with slight modifications. Thus, extraction of eDNA was done using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Amplification and quantification of eDNA were performed using the real-time PCR system (Applied Biosystems® StepOnePlus™, Thermo Fisher Scientific, Waltham, MA, USA).

Prior to extraction, a reagent mix was prepared by mixing 200 μL Milli-Q-water, 100 μL Buffer AL, and 10 μL proteinase K per sample extraction in a centrifuge tube. Water filters were folded into cylindrical form using forceps and placed in the upper part of spin columns (EconoSpin GDI-EP-31201-250, Funakoshi, Japan), which had their silica gel membranes removed prior to the input. The columns were then centrifuged at 6000*g* for 1 min to remove excess moisture from the filters.

Extraction was done by dispensing 310 μL of the reagent mix onto the filter in each spin column and the columns incubated at 56°C for 30 min. After incubation, the columns were centrifuged at 6000*g* for 1 min to collect eDNA. The eluted filtrate was then transferred to a new collection tube. Residual eDNA on each filter was collected by adding 200 μL Tris–ethylenediaminetetraacetic acid (Tris–EDTA) buffer to the filter, allowing it to stand for 1 min at room temperature (20–25°C), and centrifuging again in a new collection tube at 6000*g* for 1 min. The upper part of the column containing the filter was thereafter removed and discarded, whereas the filtrate was returned to the column holding the first filtrate by pipetting. Then, 100 μL of Buffer AL and 600 μL of ethanol were added to the pooled filtrate and mixed gently by pipetting. eDNA in each mixture was concentrated using the DNeasy Blood & Tissue Kit, according to the manufacturer's instructions. The whole amount of each mixture was transferred to a column provided in the DNeasy Blood & Tissue Kit. The column was then centrifuged at 6000*g* for 1 min and the filtrate discarded (eDNA fragments are trapped on the silica gel membrane of the column). This was done twice due to the large volume of the mixture and to ensure that all eDNA was collected. The membrane in the column was washed twice with 500 μL of Buffer AW1 and Buffer AW2 while centrifuging and discarding the filtrate following each wash. Centrifuging was done first at 6000*g* for 1 min and then 20,600*g* (max) for 2 min after washing with Buffer AW1 and Buffer AW2, respectively. eDNA was finally eluted from the columns by using 200 μL of Buffer AE and centrifuging at 6000*g* for 1 min. eDNA was stored in Lobind tubes at −20°C until quantitative PCR (qPCR) analysis.

eDNA quantification was performed using the Real-Time TaqMan® qPCR with the StepOne-Plus real-time PCR system (Applied Biosystems® StepOne-Plus™, Thermo Fisher Scientific). Amplification and quantification of the mitochondrial D-loop gene 129-bp fragments were done using primers and a TaqMan probe designed by Yamanaka et al. (2018): Oun_Dlp_Forward (5′-CATTTCCTTGCCAGGCTTAATAATA-3′), Oun_Dlp_Reverse (5′-GCAAAAGGGGGCATATATATAAGAGA-3′), and Oun_Dlp_Probe (5′-FAM-**.C.**ATAT**.G.**TTTAT**.C.**T**.C.**AT**.G.**T**.G..C.**ATAA**.C.**-TAMRA-3′), respectively. The **.C.** and **.G.** (in bold) in the probe indicate locked nucleic acids that increase melting temperature. Specificity of the primer-probe set has been previously confirmed by Yamanaka et al. (2018) through PCR using tissue samples from three fish species, namely *Opsariichthys platypus*, *Nipponocypris temminckii*, and *Nipponocypris seiboldoii*, most closely related to Hasu that occupy the same region. Each TaqMan® reaction contained 900 nM of each primer, 125 nM TaqMan® probe in the PCR master mix (TaqMan® Environmental Master Mix 2.0, Thermo Fisher Scientific), 0.075 μL AmpErase® Uracil *N*-glycosylase (Thermo Fisher Scientific), and 2 μL of the DNA template. Total volume of each reaction mixture was 15 μL. The PCR conditions were as follows: 2 min at 50°C, 10 min at 95°C followed by 55 cycles of 15 s at 95°C, and 60 s at 60°C. qPCR was performed in triplicate for each eDNA sample, and the average of each triplicate was treated as the final number of eDNA copies in the sample. The quantification of the number of Hasu D-loop genes in each 2 μL eDNA template was performed using a standard curve. A dilution series of standards containing 30, 300, 3000, 30,000, and 300,000 copies of the target sequences was used in triplicate for each qPCR assay. The standards, in which the target sequence was cloned using pEX-K4J1 vector, were provided by a commercial service (Standard Genes, Eurofins Genomics K.K., Tokyo, Japan). In this study, "undetermined" results from the quantification were treated as "0." Therefore, the final eDNA quantity in each sample was determined as an average of its 2 μL eDNA template replicates. Following Maruyama et al. (2018), no arbitrary limits of detection and quantification were set—all positive quantification data were included in the statistical analyses.

2.4 | Data analysis

All data analyses were conducted in R ver. 4.0.3 software. Plots showing the distribution of Hasu eDNA copy numbers and visual counts across the reproductive season were created for each of the 32 tributaries of Lake Biwa using the "ggplot2" and "ggpubr" packages in R.

The relationship between eDNA copy numbers and fish counts (by visual inspection) at the river channel sites was assessed using the "countreg::hurdle()" function in R. The function produces a count model and a zero-hurdle model which are suitable for zero-inflated data. A quick inspection of the data revealed that 70.6% of the visual count data had zeros (120 zero data points out of 170 total data points). This indicated zero-inflation and justified the use of a zero-hurdle model. Unlike standard count models, hurdle models are based on Bernoulli probability. In hurdle models, there is only a binary outcome of a count variate, that is, either a zero or nonzero result. If the result is nonzero, then the hurdle is crossed, and the conditional distribution of the nonzero data is regulated by a truncated-at-zero count data model (Dalrymple et al., 2003). Different combinations of $\log_{10}$ transformed eDNA copy numbers and the environmental factors (i.e., pH, electrical conductivity, turbidity, type of substrate, river velocity, depth, temperature, and width) were intuitively added to the model. During preanalysis, a strong correlation was observed between temperature and pH at the river channel sites (correlation coefficient, $r = 0.87$). The selection between the temperature and pH models favored the pH model due to its lower Akaike information criterion (AIC) score. However, it is worth noting that temperature can affect the distribution of Hasu and its eDNA. In fact, the effect of temperature on eDNA degradation, distribution, and quantity has been documented in several studies, including those by Jo et al. (2019), Bedwell and Goldberg (2020), and Kasai et al. (2020). The impact of varying pH levels on eDNA copy numbers was visually represented by modifying the pH values to correspond to the 25th, 50th, and 75th percentiles from the study in the generated model.

The effect of environmental factors on both eDNA copy number and visual count was individually assessed for river channel sites and river mouth sites (no visual counts were obtained at the river mouth in this study). Except for the correlation temperature and pH at the river channel sites (correlation coefficient, $r = 0.87$), no other strong correlations were observed among the explanatory variables. The correlation coefficients ranged from 0.51 (for depth and width in the river channel) to −0.43 (for presence of sand and presence of clay at the river mouth). Therefore, the explanatory variables were treated as independent from each other. The zero-hurdle model was not used to assess this relationship due to the large number of integer values in eDNA data. In addition, eDNA copy numbers were estimated from C_t values, indicating that they are not true count values. We acknowledge that using mixing models, such as the generalized linear mixed model, would have revealed more effects of environmental factors in the models due to the random effect from rivers. However, mixing models

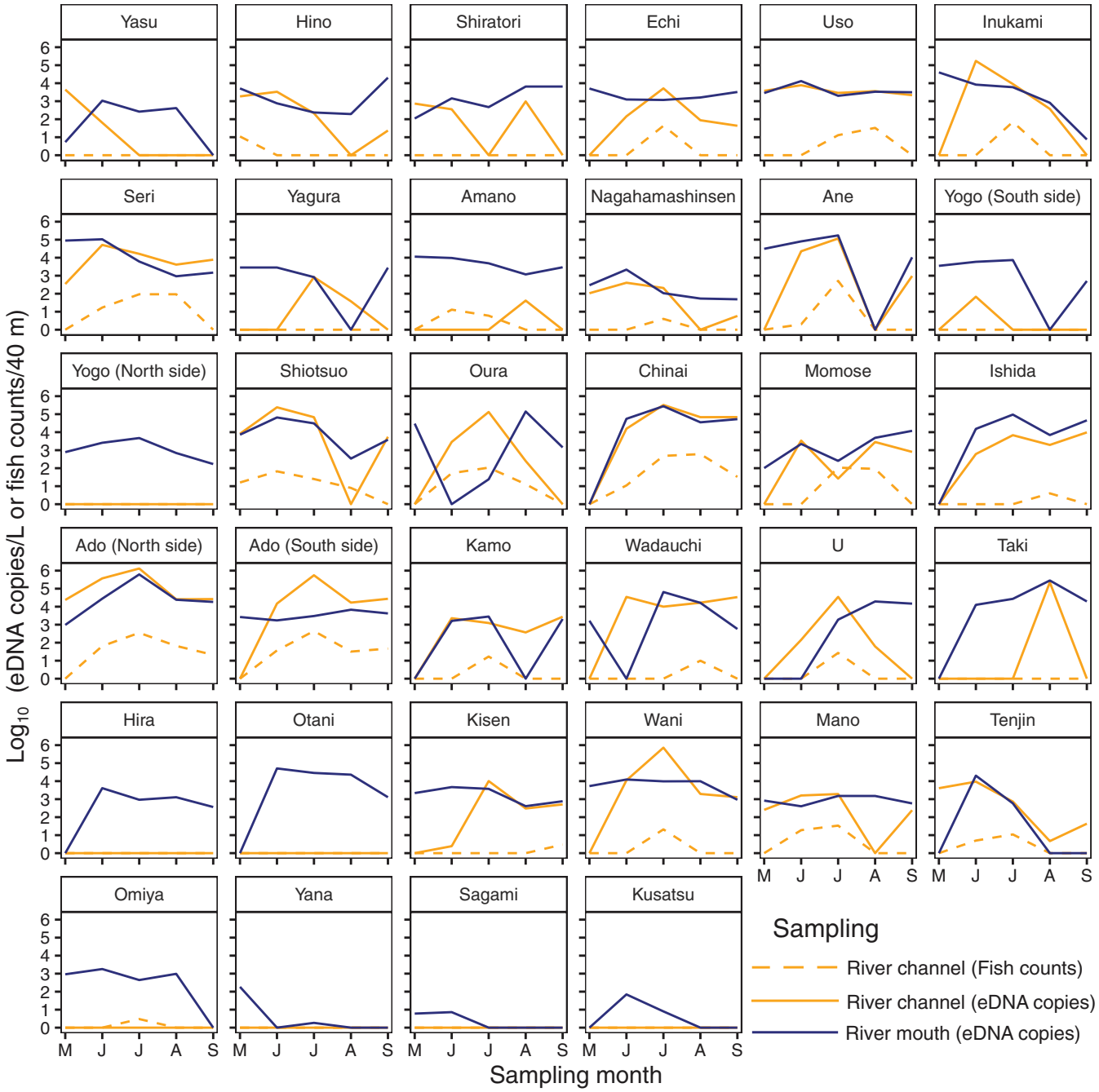

FIGURE 3 Changes in Hasu abundance in Lake Biwa tributaries from May to September revealed by environmental DNA (eDNA) analysis ($\log_{10}$, copies/L) at the river mouth sites and within the river channel sites and fish counts within the river channel sites ($\log_{10}$, per 40-m stretch). The solid blue, solid orange and orange dashed lines indicate: eDNA copies at the river mouth, eDNA copies within the river channel and fish counts within the river channel sites, respectively.

could not be applied in the present study due to an insufficient number of datapoints (34 rivers × 2 sampling sites × 5 months × average of 3 replicates for each measurement = 340 records). This study is the first large-scale study on Hasu reproductive migration; as a result, data from all the rivers were pooled to effectively assess the effects of environmental factors on the models.

The analysis was therefore done in two steps. First, the effects of environmental factors on the presence of Hasu and its eDNA were assessed using generalized linear models (GLMs) with a binomial logit function. This was implemented by the "base::glm()" function in R. Variable selection for the final model was then achieved through forward and reverse stepAIC and by using the "base::stepAIC()" function in R. In the second part of the analysis, linear models (LMs) built with the "base::lm()" function, were used to assess the relationship between positive eDNA copy numbers and visual counts

TABLE 1 Coefficients (estimates ± standard errors) for the zero-hurdle model, determined after AIC selection, to estimate individual numbers from eDNA copies and environmental factors.

	Count model[a]	Hurdle model[b]
(Intercept)	$1.45 \times 10^{0} \pm 4.24 \times 10^{-1}$***	$-2.83 \times 10^{0} \pm 4.28 \times 10^{-1}$***
eDNA ($\log_{10}$, copies/L)	$4.17 \times 10^{0} \pm 1.50 \times 10^{0}$***	$4.44 \times 10^{0} \pm 1.48 \times 10^{0}$**
eDNA ($\log_{10}$, copies/L): pH	$-4.71 \times 10^{-1} \pm 1.50 \times 10^{-1}$**	$-4.72 \times 10^{-1} \pm 1.91 \times 10^{-1}$*
Log (theta)[c]	$-5.15 \times 10^{-1} \pm 2.85 \times 10^{-1}$	

Note: Only datasets from river channel sites were used since no visual inspections were conducted at the river mouth.

Abbreviations: AIC, Akaike information criterion; eDNA, environmental DNA.

[a]Count model assumed truncated negative binomial distribution and used log link function.

[b]Hurdle model assumed binomial distribution and used a logit link function.

[c]Theta is a measure of overdispersion with respect to the Poisson distribution. Theta N_0: Theta = 1, that is, there is no excess zeros in the data.

Significance levels by Wald tests: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

as response variables and environmental factors as explanatory variables. Variable selection for the final LMs was also achieved by forward and reverse stepAIC. In all cases, the model with the least AIC score was selected. The effect of depth on eDNA copy numbers at the river mouth was not assessed due to difficulties in collecting information on depth.

3 | RESULTS

3.1 | Distribution of Hasu and its eDNA in Lake Biwa tributaries across the reproductive season

Hasu eDNA was detected in all 32 rivers at some point during the reproductive season (Figure 3). Except for a few mismatches (eDNA not obtained despite visual observation; $n = 5$ out of 50 datasets, derived from an eDNA sample and visual presence at each sampling in the entire study), Hasu eDNA was detected in sites where the fish was visually observed during each sampling. Importantly, there was no amplification of Hasu eDNA in any negative controls prepared in the field as well as those used in qPCR analysis—implying that the effect of inter-sample contamination was negligible.

The R^2 values for the qPCR tests ranged from 0.95 to 0.99 (qPCR efficiency: 64.69%–88.68%). The most individual counts ($n = 605$ out of 3756 total fish counts in the entire study), in one sampling of Hasu at a sampling site, were obtained within the month of August in the Chinai River located on the north-western side of Lake Biwa (Figure 3). The highest number of eDNA copies (1.34×10^{6} copies/L) were also obtained on the north-western side of Lake Biwa in the northern arm of Ado River within the month of July (Figure 3). In some months and at certain sites, especially on the south-western side of Lake Biwa (i.e., Hira, Omiya, and Taki rivers), no eDNA analysis or visual inspection was conducted due to river drying.

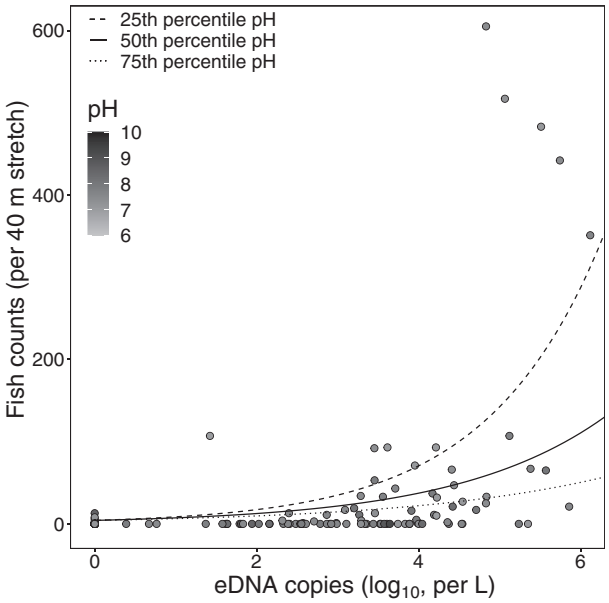

FIGURE 4 Relationship between eDNA copies at sites within the river channel ($\log_{10}$, /L) and fish counts from visual inspection (per 40-m stretch) in the river channel, as revealed by the zero-hurdle model. dashed, solid, and dotted lines indicate the equations at the 25th percentile pH (7.37), 50th percentile pH (7.71), and 75th percentile pH (7.99), respectively.

3.2 | Relationship between Hasu eDNA copies and visual counts

The zero-hurdle model was used to assess the relationship between visual counts and eDNA copies for only the river channel sites (Table 1). No visual count data were obtained at the river mouth due to difficulties in collecting information. The model revealed that estimating the number of Hasu individuals from eDNA copy numbers

TABLE 2 Presence or absence of Hasu eDNA copies (determined through eDNA analysis) and presence or absence of Hasu fish (determined by visual inspection), evaluated in relation to environmental factors as explanatory variables using GLMs with coefficients (estimates ± standard errors) selected via stepAIC.

	Presence or absence of Hasu eDNA		Presence or absence of Hasu fish[a]
Coefficients	**River mouth**	**River channel**	**River channel**
(Intercept)	$-4.93 \times 10^{0} \pm 2.15 \times 10^{0}$*	$-2.51 \times 10^{0} \pm 1.02 \times 10^{0}$*	$-1.79 \times 10^{0} \pm 5.18 \times 10^{-1}$***
Presence of gravel	$2.57 \times 10^{0} \pm 8.24 \times 10^{-1}$**		$1.22 \times 10^{0} \pm 4.85 \times 10^{-1}$*
Presence of sand	$1.85 \times 10^{0} \pm 7.93 \times 10^{-1}$*		
Presence of clay			
River width (m)	$8.61 \times 10^{-3} \pm 5.57 \times 10^{-3}$	$3.26 \times 10^{-2} \pm 1.38 \times 10^{-2}$*	$3.68 \times 10^{-2} \pm 1.40 \times 10^{-2}$**
River depth (m)			
Electrical conductivity (μS/cm)			$-4.29 \times 10^{0} \pm 2.49 \times 10^{0}$
Turbidity (log10, NTU)		$-2.31 \times 10^{-2} \pm 1.51 \times 10^{-2}$	$-9.10 \times 10^{-2} \pm 6.06 \times 10^{-2}$*
pH		$2.62 \times 10^{-1} \pm 1.32 \times 10^{-1}$*	
Water velocity ($\log_{10}(x+1)$, cm/s)		$9.81 \times 10^{-3} \pm 4.49 \times 10^{-3}$*	$8.91 \times 10^{-3} \pm 5.02 \times 10^{-3}$
Water temperature (°C)	$1.69 \times 10^{-1} \pm 7.73 \times 10^{-3}$*		

Note: For presence or absence of eDNA copies versus environmental factors, river mouth and river channel sites were assessed independently. For presence or absence of fish by visual inspection vs environmental factors, only datasets from the river channel sites were used since no visual inspections were conducted at the river mouth. GLMs assumed binomial distribution and used a logit link function.

Abbreviations: AIC, Akaike information criterion; eDNA, environmental DNA; GLM, generalized linear models.

[a]By visual inspection.

Significance levels using Wald tests: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

was positively influenced by the amount of $\log_{10}$ transformed eDNA copies ($p < 0.05$). However, when environmental factors were included in the model, the interaction term between $\log_{10}$ transformed eDNA copies and pH (pH range: 6.86–9.50) had a significant reducing effect on the estimated number of Hasu individuals derived from eDNA copies ($p < 0.05$; Figure 4).

3.3 | Effect of environmental factors on Hasu eDNA copies and visual counts

At the river mouth, using stepAIC in the GLM model, we identified gravel and sand presence, river mouth width, and water temperature as factors influencing the presence of eDNA copies at sampling sites (Table 2). However, only gravel and sand presence, along with temperature, showed significant positive effects ($p < 0.05$). In addition, in the LM, gravel presence in the river and water current velocity positively influenced the number of eDNA copies obtained at sampling sites, while electrical conductivity had a negative impact (Table 3 and Figure 5, Adj. R^2: 0.18, $p < 0.05$). At the river channel sites, using stepAIC, we identified river width, turbidity, pH, and current velocity as factors influencing the presence of eDNA at sampling sites (Table 2). Nevertheless, all factors, except for turbidity, had positive and significant effects in the assessment ($p < 0.05$). In the second part of the analysis, only gravel presence had a positive and significant influence on the model, while pH and water turbidity had negative but significant impacts on the number of eDNA copies obtained at the sampling sites (Table 3 and Figure 6, Adj. R^2: 0.16, $p < 0.05$).

For the presence of Hasu at river channel sites, using stepAIC, we identified gravel presence, river width, electrical conductivity, turbidity, and current velocity as factors influencing fish presence (Table 2). However, only gravel presence and river width had a positive and significant effect in the assessment, while turbidity had a negative but significant impact on the model ($p < 0.05$). In the LM assessing the impact of environmental factors on visual counts, only river depth had a positive effect on the model, while pH had a negative but significant impact on the number of Hasu at the sampling sites (Table 3 and Figure 7, Adj. R^2: 0.21, $p < 0.05$).

4 | DISCUSSION

4.1 | Significance of using complementary methods on Hasu migration ecology

We found positive correlation between the number of Hasu eDNA copies and counts through visual inspection

TABLE 3 Positive observations (>0) of Hasu eDNA copies (determined through eDNA analysis) and positive visual counts (>0) of Hasu fish (determined by visual inspection), evaluated in relation to environmental factors as explanatory variables using simple LMs with coefficients (estimates ± standard errors) selected via stepAIC.

	Positive Hasu eDNA copies (log_{10}, copies/L)		Positive visual counts (log_{10}, per 40 m)
Coefficients	**River mouth**	**River channel**	**River channel**
(Intercept)	$4.96 \times 10^{0} \pm 1.54 \times 10^{0}$**	$7.38 \times 10^{0} \pm 2.03 \times 10^{0}$***	$5.82 \times 10^{0} \pm 1.95 \times 10^{0}$**
Presence of gravel	$6.02 \times 10^{-1} \pm 1.72 \times 10^{-1}$***	$5.35 \times 10^{-1} \pm 2.62 \times 10^{-1}$*	
Presence of sand	$3.36 \times 10^{-1} \pm 2.01 \times 10^{-1}$		
Presence of clay			
River width (m)			$6.06 \times 10^{-1} \pm 2.46 \times 10^{-1}$*
River depth (m)		$6.07 \times 10^{-1} \pm 3.37 \times 10^{-1}$	
Electrical conductivity (μS/cm)	$-4.79 \times 10^{0} \pm 1.51 \times 10^{0}$**		$-2.36 \times 10^{0} \pm 1.34 \times 10^{0}$
Turbidity (log10, NTU)		$-1.01 \times 10^{0} \pm 2.98 \times 10^{-1}$**	
pH	$-2.62 \times 10^{-1} \pm 1.80 \times 10^{-1}$	$-5.50 \times 10^{-1} \pm 2.65 \times 10^{-1}$*	$-5.89 \times 10^{-1} \pm 2.62 \times 10^{-1}$*
Water velocity ($log_{10}(x+1)$, cm/s)	$6.60 \times 10^{-1} \pm 2.71 \times 10^{-1}$*		
Water temperature (°C)			

Note: For positive number of eDNA copies versus environmental factors, river mouth and river channel sites were assessed independently. For individual counts versus environmental factors, only datasets from river channel sites were used since no counts by visual inspection were obtained at the river mouth.
Abbreviations: AIC, Akaike information criterion; eDNA, environmental DNA; GLM, generalized linear models; LMs, linear models.
Significance levels using Wald tests: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

(Figure 4 and Table 1). Maruyama et al. (2018) previously demonstrated a similar positive correlation between the number of Hasu eDNA copies and counts through visual inspection. However, due to the limited sample size, specifically data obtained from a single river, Maruyama et al. (2018) were unable to statistically account for the effect of environmental factors on this relationship. By using a zero-hurdle model and data from the 32 rivers, this study was able to account for the effect of environmental factors on the relationship between Hasu eDNA copies and counts through visual inspection. Thus, the number of migrating Hasu individuals in the 32 river channel sites, within a 40-m stretch upstream from an eDNA sampling point, can be best estimated using eDNA copy numbers (log_{10}) from a 1 L sample and the interaction term between the eDNA copy numbers (log_{10}) and the pH of the water sample. This can be expressed as an equation as follows:

$$N_{\text{Hasu}} = 4.44 \times \log_{10}[\text{eDNA}] + 0.47 \times \log_{10}[\text{eDNA}] \times \text{pH} - 2.83,$$

where N_{Hasu} is the number of estimated Hasu in the 40-m stretch upstream of an eDNA sampling point; [eDNA] is the number of eDNA copies in a liter of the river water sample and pH is the pH of the water at the sampling site.

The introduction of pH into the relationship between Hasu eDNA copies and counts through visual inspection represents progress in the fields of eDNA analysis and assessment tools for identifying Hasu migration hotspots. However, if not carefully considered, it could lead to an underestimation of the number of migrating Hasu in the rivers. In this study, the average pH in the river channel sites was high at the beginning of the reproductive season, i.e., in May (mean pH = 8.32 ± 0.54) and decreased with progression of the reproductive season, with the lowest pH being recorded in August (mean pH = 7.53 ± 0.40), before slightly increasing again at the end of the reproductive season, that is, in September (mean pH = 7.66 ± 0.31). As the pH increases, the relationship curve between eDNA copies and counts by visual inspection flattens (Figure 4 and Table 1) and moves closer to zero. Therefore, at high pH levels, estimating individual counts from eDNA copies becomes difficult, leading to increased uncertainty and possibly underestimation.

Although DNA is relatively stable in alkaline solutions, it may denature at extremely high pH because some hydrogen bond acceptors involved in base pairing become protonated (Moret et al., 2001). It is also worth mentioning that this study used glass fiber filters to collect eDNA from water samples, which have been documented to have low collection efficiency in alkaline solutions (Tsuji et al., 2017). This could result in an underreporting of the amount of eDNA present in the river and affect results. When possible, using complementary methods, such as counts through visual

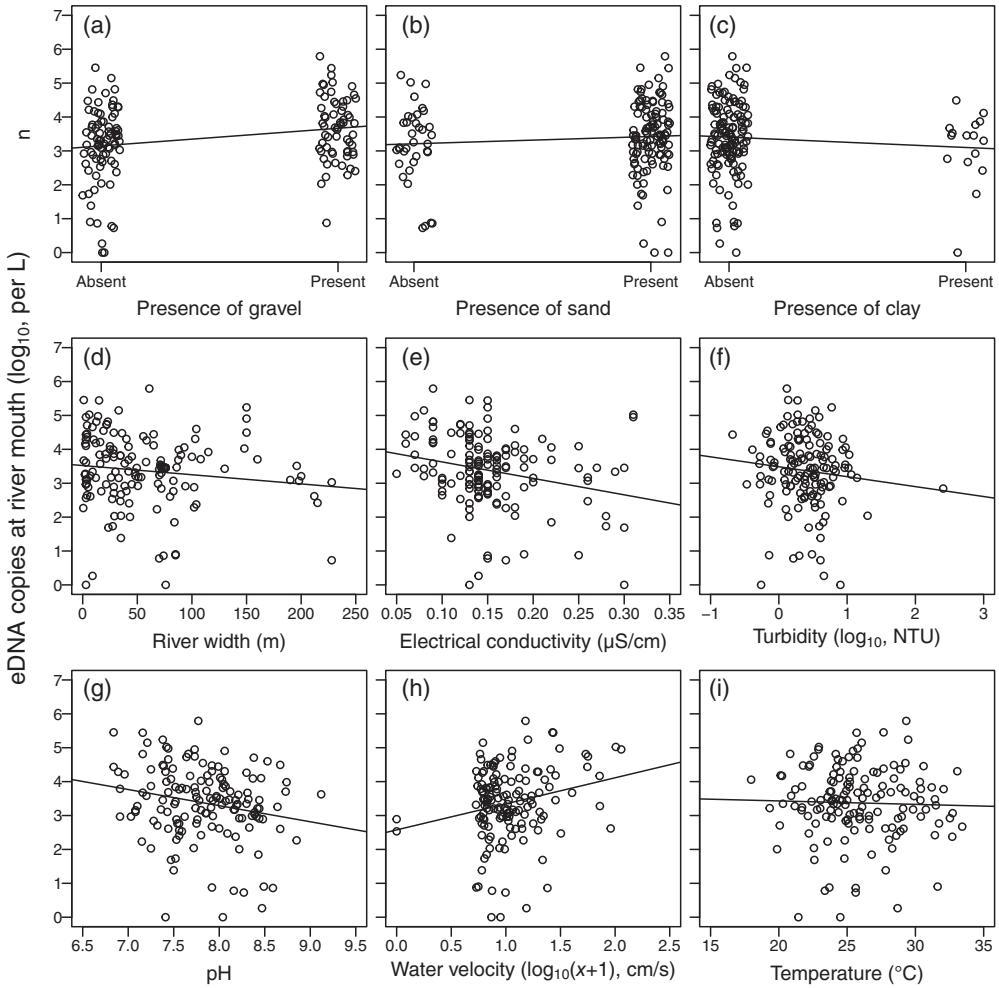

FIGURE 5 (a–i) Plots of positive eDNA observations (log_{10}, copies/L) as the response variable and environmental factors as explanatory variables at the river mouth sites. For the presence of gravel (a), sand (b), and clay (c), the binomial variables were jittered to reduce dense clustering of points during plotting.

inspection, in highly alkaline rivers and at the beginning of the reproductive season when pH levels are generally high, causing the eDNA-pH-based model to become unreliable, could help address the issue of underestimation and provide more accurate estimates of the number of Hasu migrating in the rivers.

In some rivers, especially at the river mouth, visual inspection was difficult due to factors such as high turbidity, increased depth, greater width, and the absence of bridges. In these rivers, eDNA analysis provided a more effective tool for detecting the presence or absence of migrating Hasu, primarily because of the ease in collecting water samples. The use of complementary methods in this study provided a holistic approach to better understand the migration patterns of Hasu in the 32 tributaries of Lake Biwa. The choice on which method to use at a sampling point rests entirely on the researcher's judgment. When using complementary methods, considering characteristics of the rivers and resources available to the researcher can help determine the most suitable method to use for Hasu migration assessment.

4.2 | Migration hotspots of Hasu during the reproductive season and their characteristics

This study revealed that Hasu is widely distributed in the inlet tributaries of Lake Biwa during the reproductive season, with a greater presence in tributaries on the north-western side of Lake Biwa. Both eDNA analysis and visual counting showed that Hasu was abundant in rivers that had gravel or sandy substrates, were fast flowing at the river mouth, were less turbid, had reduced alkalinity, and were deep and wide enough to accommodate migrating individuals. These findings are consistent with previous observations by Tanaka (1964) and a study along the shoreline of Lake Biwa by Imamura (2018). The largest migrating groups of Hasu were recorded in Chinai and Ado Rivers by visual counts and eDNA copy numbers, respectively (Figure 3). Both rivers are located on the northern-western side of Lake Biwa. This region has fewer human settlements and is characterized by mountain ranges relatively close to the lake compared to the eastern side. The Ado River, which has two arms

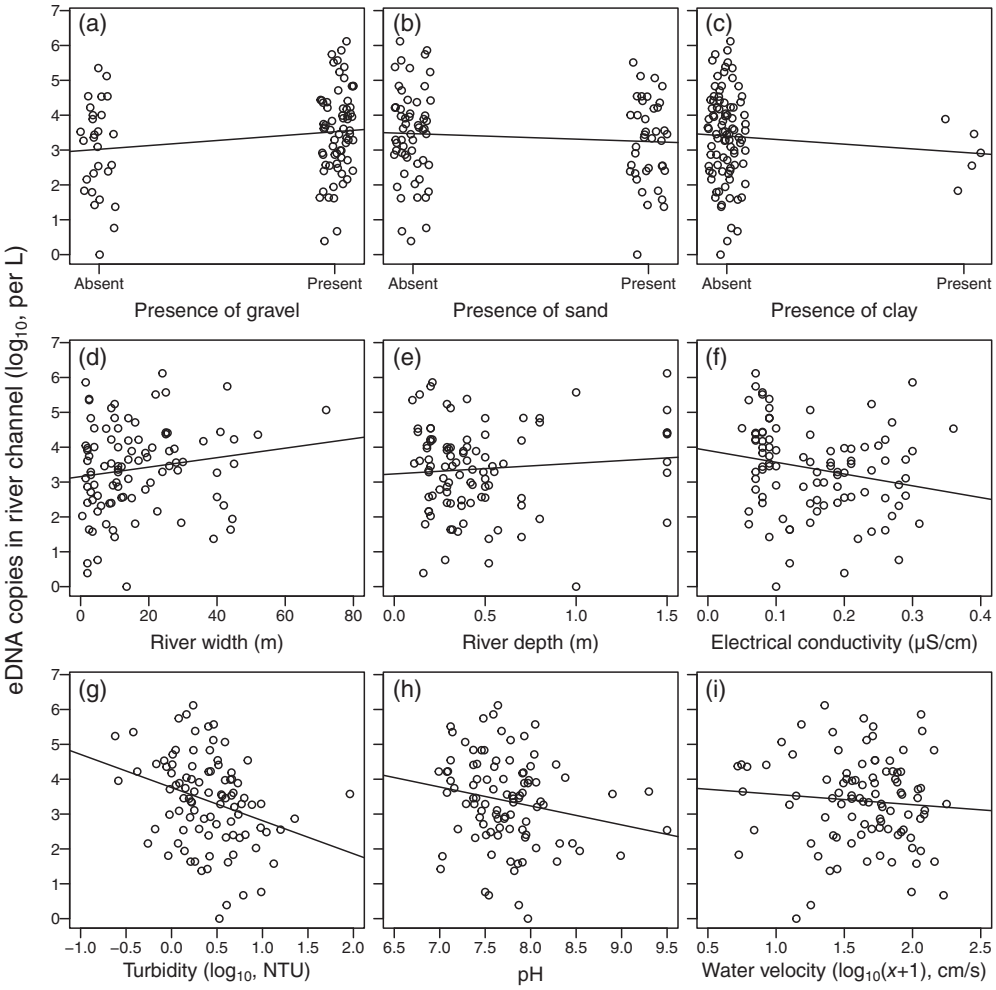

FIGURE 6 (a–i) Plots of positive eDNA observations (log_{10}, copies/L) as the response variable and environmental factors as explanatory variables within the river channel. For the presence of gravel (a), sand (b), and clay (c), the binomial variables were jittered to reduce dense clustering of points during plotting.

feeding into Lake Biwa, had more migrations in the northern arm than in the southern arm. Additional assessment of the two river arms is needed to understand the variation in Hasu densities.

Ado, Chinai, Seri, and Shiotsuo Rivers, all located in the northern basin of Lake Biwa, exhibited similar trends in both eDNA copy numbers and visual counts. In these rivers, eDNA copies and visual counts increased from May to July during the reproductive season and decreased from August to September. This distinct change in fish counts and eDNA copies, with progression of the reproductive season, is indicative of recently migrating Hasu. Ado, Chinai, Seri, and Shiotsuo Rivers are characterized by gravel and/or sandy substrates, do not dry out during the reproductive season, and are sufficiently deep for upstream migration. These rivers, with their specific characteristics, are important for Hasu migration and can be considered as Hasu migration hotspots. In contrast, Hasu were not visually observed in most rivers in the southern basin. No eDNA and visual counts were obtained at the river channel sites in Hira, Otani, Omiya, Yana, Sagami, and Kusatsu Rivers. Several factors contribute to these observations, including drying up during the reproductive season in some rivers (e.g., Otani, Hira, and Omiya Rivers), inadequate depth to accommodate Hasu upstream migration in others (e.g., Sagami and Yana Rivers), and possibly the redirection and modification of these rivers to meet human needs in recent history (e.g., Yogo River in the northern basin and Kusatsu River in the southern basin; Ministry of Land, Infrastructure, Transport and Tourism, Japan, 2016a, 2016b). There was also little or no eDNA at the river mouth in Yana, Sagami, and Kusatsu Rivers. This implies that there were fewer or no Hasu approaching these rivers.

Compared to the north-western side, there are fewer Hasu migrations on the other sides of Lake Biwa, which are more urbanized, and the water quality is poorer, especially in the southern basin. This could be a result of agricultural inputs from surrounding paddy fields and high population density in the regions (Shiga Prefectural Government, 2018; Yoshioka, 1991). Extreme changes in pH due to agricultural inputs have been documented to create toxic environments for some freshwater fish

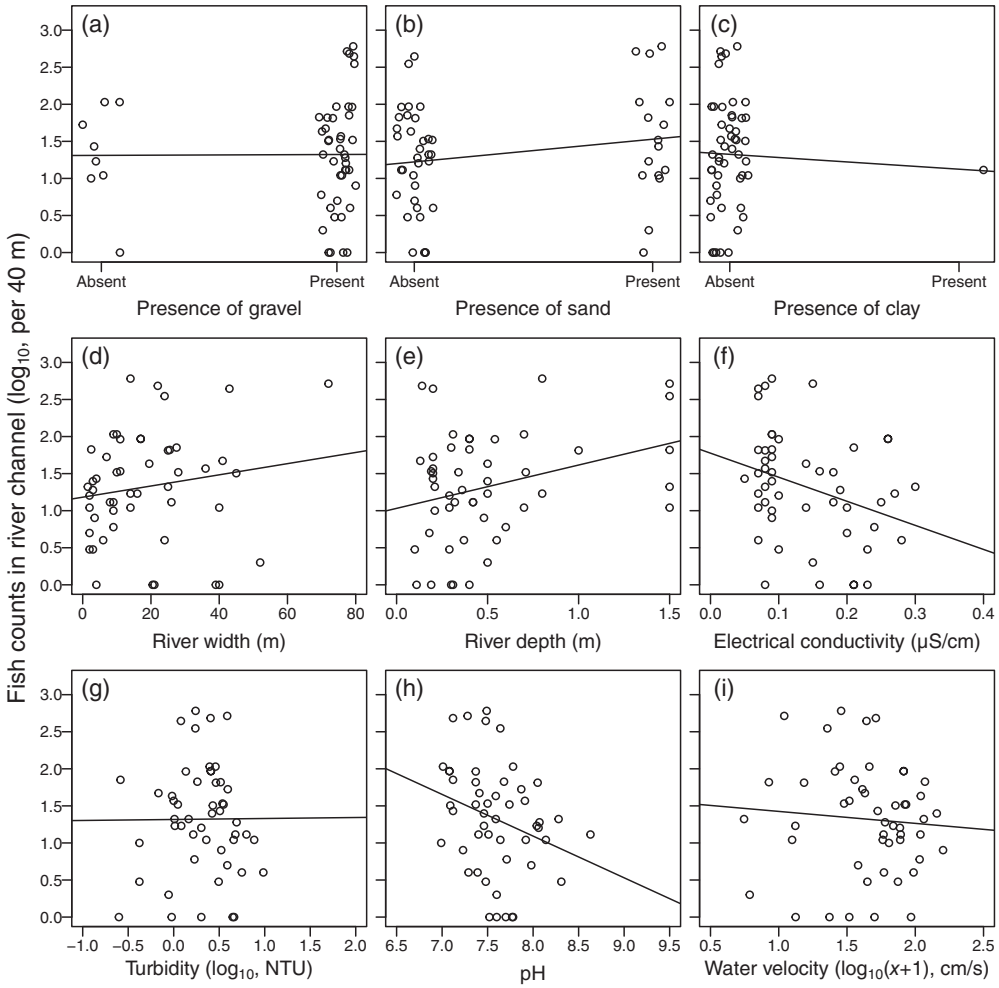

FIGURE 7 (a–i) Plots of positive visual encounters (log_{10}, counts/40 m) as the response variable and environmental factors as explanatory variables within the river channel. For the presence of gravel (a), sand (b), and clay (c), the binomial variables were jittered to reduce dense clustering of points during plotting.

elsewhere (Zhou & Boyd, 2014). In addition, land reclamation in the 1960s has led to significant modifications on the coastline of Lake Biwa (Zeballos & Yamaguchi, 2011). The water quality in Lake Biwa deteriorated significantly in the 1960s due to rapid population growth, inadequate wastewater treatment, and agrochemical abuse, all of which are key drivers of eutrophication in lakes (Kita et al., 2006). Some rivers have been redirected, while others reconstructed to accommodate human needs. Reconstruction has also seen the introduction of concrete riverbanks and substrates in some rivers (e.g., in Sagami River), prompting the reduction of gravel, a vital component for Hasu reproductive migration. Despite efforts by the local government to prevent further deterioration of the lake's ecosystem, ecosystem restoration has been slow—potentially explaining the fewer migrations in this region. Changes in land use and artificial modifications to rivers have the potential to reshape sediment distribution and flow velocity. Rivers that flow from the highly developed regions, such as those in the southern basin, tend to have smaller downstream gradients compared to those in less developed regions, like those the northern basin (Weilhoefer et al., 2002). It is possible that Hasu avoided migrating to the rivers with smaller downstream gradients, as our results show that Hasu tend to prefer rivers with sandy-gravel substrates and fast-flowing water.

Although visual confirmation was not possible in some turbid rivers, such as the Shiratori River located on the eastern side of the northern basin, the presence of eDNA copies indicated recent Hasu migrations—albeit in smaller quantities than in clear running rivers. Most piscivorous species, including Hasu, rely on their keen eyesight for foraging (Hori et al., 2021; Pita et al., 2015). Turbid waters, as those in Shiratori River, may affect the line of sight in Hasu, making it difficult to forage for prey species. Furthermore, piscivorous species like Hasu require higher oxygen levels due to their quick foraging movements (Hansen et al., 2013; Jackson et al., 2011). The presence of particles in murky and turbid rivers may obstruct oxygen intake in their gills, causing Hasu to avoid such rivers. If the population of Hasu is to be increased in Lake Biwa and its ecosystem, there is a need to protect rivers with clear running water, especially

rivers on the western side of the northern basin and improve the quality of water especially in the southern basin. However, further assessment is needed to understand the impacts of turbidity on prey capture in chasing fish such as Hasu.

In conclusion, this study advocates using complementary methods for least studied species such as Hasu. Our findings revealed the distribution of the potamodromous Hasu and its eDNA during its reproductive migration in 32 tributaries around Lake Biwa. The study also highlighted the significance of incorporating environmental factors such as pH into Hasu migration studies. Our eDNA analysis and visual count data revealed that Hasu migration occurs mostly in the northern basin of Lake Biwa especially in the western side. There is a need to protect rivers in this region, including rivers like the Ado River, Chinai River and Shiotsuo River, to name a few, during the reproductive season. These rivers have good water quality, fast-flowing currents, sandy-gravel substrates, are wide and deep enough to accommodate migrating Hasu and do not dry up, all of which are important for successful reproductive migration of Hasu.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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Ichthyological Research
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FULL PAPER

An assessment of the potamodromous fish *Opsariichthys uncirostris uncirostris* (Hasu fish) during its reproductive migration to a Lake Biwa tributary using stable isotope ratios and biometric data

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Abstract
Opsariichthys uncirostris uncirostris (Hasu fish), a vulnerable potamodromous fish, is the only piscivorous cyprinid fish in Japan and endemic as a subspecies to Lake Biwa. The species population is on a continued decline for the past 70 years. This study aimed at developing a portfolio on the species during its reproductive migration to Shiotsuo River, a Lake Biwa tributary, by using a combination of biometric measurements and stable isotope ratios in its tissues. Hasu fish were collected monthly, from May to September 2019, using cast nets. The biometric measurements: wet weight, standard length, gonad weight and gut content were collected and used to calculate the gonado-somatic index (GSI) and Fulton's condition constant (K) and determine the feeding habits of Hasu fish. Carbon and nitrogen isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) in slow-changing muscle and fast-changing mucus tissues of Hasu fish were also used to determine recent diet change. At the beginning of the reproductive season, fewer females than males were caught; however, the number of females increased as the season progressed. On average, males were larger than females. Migrating individuals were healthy ($K > 1$) and over 37% larger in length than those in the 1960s. Gut content analysis revealed, for the first time, Hasu fish feeding in the rivers, primarily on Ayu fish, during the reproductive migration. $\delta^{13}C$ and $\delta^{15}N$ in muscle and mucus indicated a recent change in diet, i.e., from Lake Biwa to Shiotsuo River, with differences in the onset of feeding (and consequently upstream migration) between sexes and individuals. For the effective conservation of Hasu fish in the other tributaries where Ayu fish traps block other fishes' migration, we recommend having the rivers open from June to September to cover its variable timing in the recruitment of reproductive individuals.

Keywords Riverine environment · Stable isotope analysis · Potamodromous fishes · Fulton's condition factor (K) · Feeding ecology

Introduction

Opsariichthys uncirostris uncirostris (Hasu fish) is a potamodromous fish that relies on lake–river migration for its reproduction. It is the only piscivorous cyprinid fish in Japan, endemic as a subspecies to Lakes Biwa and Mikata. Its population has been experiencing a continued decline for the last 70 years and it is considered vulnerable in Lake Biwa and extinct in Lake Mikata (Ministry of the Environment, Japan 2020). Unfortunately, the dynamics surrounding Hasu fish reproductive migration has not been extensively studied since the 1960s. The fish is reported to spawn in summer from late May to early August in the shores of Lake Biwa and its tributaries, with mostly mature males and females of ages 3 years (average body length: 160 mm) and 2 years (average body length: 130 mm), respectively (Tanaka 1964; Miura 1966). Using environmental DNA (eDNA) analysis and counts through visual inspection, Maruyama et al. (2018) and Mvula et al. (2023) were able to determine that Hasu fish abundance gradually increases in tributary rivers of Lake Biwa during the reproductive season from May to July before eventually decreasing in August and September. Even though eDNA provides a snapshot of the distribution of Hasu fish in Lake Biwa tributaries, it currently does not offer insights into the intake of nutrients and residence time

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of fish in the tributaries. These can be estimated through the feeding habits and biometrics of the migrating fish. However, the feeding habits of Hasu fish during its reproductive migration to Lake Biwa tributaries are currently unknown. These feeding and biometric assessments are important to determine the overall health status and thus aid in the conservation of Hasu fish during reproductive migration.

For decades, conventional measurements, including diet assessments and length–weight measurements, have been the gold standard for gaining insights into the feeding habits and biometrics of various migrating species. For instance, wet weight and standard length measurements have been used to provide data on individual fish size and mass, enabling us to monitor the growth and overall condition of a species (Moutopoulos and Stergiou 2002). Analyzing gut contents has also allowed for a better understanding of the feeding behavior and dietary preferences of such species during migration. In addition, indices such as the gonado-somatic index (GSI) and Fulton's condition constant (*K*) have been used to assess the timing and intensity of spawning events (Roy et al. 2014; Mozsár et al. 2015). These biometric tools can thus be used to reflect the overall health status of migrating Hasu fish and consequently aid in the conservation of the fish in Lake Biwa tributaries.

In recent years, the use of stable isotopes has also gained traction in ecological studies. A stable isotope is an element whose relative proportions do not vary over time due to the absence of radioactivity (Gill 2015). Stable isotopes remain unchanged and transfer in a predictable manner between trophic levels (Hobson et al. 1997). When a species migrates and begins to feed in a new environment, the composition of stable isotopes within its tissues changes, reflecting the isotope ratios of the diet in the new environment (Phillips and Eldridge 2006). Different tissues have different turnover rates, i.e., the rate at which the tissues change to reflect the isotope ratios in the new environment (Hobson and Clark 1992). For example, in fish, mucus tissues tend to have faster turnover rates when compared to dorsal muscle and fin tissues (Maruyama et al. 2016; Shigeta et al. 2017; Winter et al. 2019).

By taking advantage of the differences in turnover rates in the two tissues (e.g., the slow change in muscle and the fast change in mucus tissues), and on the likely assumption that the migrating animals are in isotopic equilibrium with its former environment when they start migration, migration researchers can roughly estimate the time when a species changed its diet, and consequently its environment from one sampling (Phillips and Eldridge 2006; Heady and Moore 2013). It is important to emphasize that the environmental equilibrium values may vary between tissues due to the inherent differences in stable isotopes and their specific diet-tissue discrimination factors (i.e., the differences between a tissue's stable isotope ratio and that of its diet when in isotopic equilibrium). For instance, using adult *Silurus asotus* as a model species in a diet switch experiment, Maruyama et al. (2016) demonstrated that the initial stable carbon isotope ratios were higher in muscle tissue than in mucus tissue. Regardless of the differences in equilibrium values and even though stable isotope ratios in the tissues change at different rates, it is assumed that they (stable isotope ratios in different tissues) are bound to reach new equilibriums with a consistent diet in the new environment. The two commonly used stable isotopes in ecological studies are carbon and nitrogen isotope ratios (hereafter, $\delta^{13}C$ and $\delta^{15}N$, respectively), which exhibit a stepwise increase of about 1‰ and 2.5–5‰ at each trophic level, respectively (DeNiro and Epstein 1978, 1981; Minagawa and Wada 1984; Hobson et al. 1997; Post 2002). These two stable isotopes play a crucial role in understanding the transfer of carbon and nitrogen through the food web in different ecosystems.

Generally, lake ecosystems tend to have lower $\delta^{13}C$ and higher $\delta^{15}N$ when compared to river ecosystems (Le Bourg et al. 2018; Wilkinson et al. 2022). This is largely due to the differences in reaction rates between phytoplankton and periphyton. In the Lake Biwa ecosystem, in particular, $\delta^{13}C$ have been documented to be higher in tributary rivers than in the lake, with zooplankton in the lake exhibiting lower $\delta^{13}C$ than benthic invertebrates in the tributaries (Yamada et al. 1998; Maruyama et al. 2001; Sawada et al. 2019). Using eggs in ovaries of fish, Ito et al. (2015) and Sawada et al. (2019) also independently demonstrated that $\delta^{15}N$ are higher in Lake Biwa than in its tributaries, reflecting the differences between primary producers and basal animals (Yamada et al. 1998). Thus, it is expected that Hasu fish migrating from Lake Biwa and feeding in the tributaries will exhibit $\delta^{13}C$ and $\delta^{15}N$ changes in its tissues to reflect those of the river environment over a period of time.

By integrating data from the conventional biometric measurements and stable isotope ratios, we can develop a more comprehensive portfolio on Hasu fish populations during their reproductive migration. Therefore, the aim of this study was to assess the feeding habits and residential time of migrating Hasu fish, in a Lake Biwa tributary, by using conventional biometric measurements and examining stable isotope ratio changes in its mucus and muscle tissues across its reproductive season. This integrated approach should facilitate conservation efforts and the adoption of sustainable practices to ensure the long-term viability of Hasu fish and the preservation of its ecosystem.

Materials and methods

Description of study site. The study was conducted in the lower reaches of the Shiotsuo River, flowing into Lake Biwa from the north. The river has a total length of 9 km and a

basin area spanning 21.8 m^2 (Ministry of the Environment, Japan 2023). The river primarily flows through mountainous terrain (95.8% of the total river catchment area), contributing to a steep gradient of 9.5 m/km, making it one of Lake Biwa's steepest rivers and a preferred reproductive upstream migration route for Hasu fish. Due to its steep gradient, the river is fast-flowing, and it is also characterized by gravel bottom substrates, all of which have been identified to be key drivers of Hasu reproductive migration (Mvula et al. 2023). In addition to the ease of sample collection, these characteristics are why Shiotsuo River was selected as a study site in this study.

The river is perennial, and there are no high weirs in the middle and lower reaches of the river, allowing for natural upstream migration of Ayu fish, as well as Hasu fish. Ayu fish serves as the primary food source for Hasu fish in Lake Biwa (Tsunoda et al. 2015), and possibly in the tributaries, but the feeding habits of Hasu fish in rivers during reproductive migration remains unknown. Abundant natural Ayu fish in the river also attracts recreational fishermen, but fishing is not permitted in the lower reaches, where Ayu spawning parents are released approximately 1 km upstream from the river mouth. The river also has a protected spawning area that extends 4.5 km from the river mouth. These characteristics make Shiotsuo River a conducive environment for Hasu fish reproductive upstream migration.

Fish sampling and biometric measurements. Hasu fish samples were collected monthly in the lower reach (2–3 km from the river mouth) of Shiotsuo River from May to September 2019 using cast nets. The sampling was systematic and a target of 20 Hasu individuals was set for each sampling. Wet weight (g), standard length (mm), and gonad weight (g) were measured (to the nearest 0.01 g or 0.1 mm) in the field. The species composition of the gut content was also recorded in the field. The gut contents of each fish, and the fish itself, were put in individual Ziploc® bags to avoid inter-sample contamination and kept on ice in a cooler box while in the field. After the field sampling, samples were transported to the laboratory at Ryukoku University and kept frozen below −22.5 °C until analysis. The Fulton's condition constant (K) and gonado-somatic index (GSI) were also calculated for each fish using the following formulas, respectively:

$$K = 100 \times \frac{W}{L^3},$$

where W (g) is the wet weight of the fish and L (cm) is the standard length of the fish.

$$GSI = 100 \times \frac{W_g}{W_b},$$

where W_g (g) is the gonad wet weight and W_b (g) is the body wet weight of the fish.

Multi-tissue stable isotope analysis. At the laboratory, epidermal mucus was wiped directly along the lateral line on the left body surface of each thawed specimen using quarter of a 25 mm-diameter GF/F glass microfiber filter (GE Healthcare, Buckinghamshire, UK). Each filter was oven dried at 60°C for 48 h and cleaned using forceps to remove scales or skin fragments if present (as done in previous studies, e.g., Maruyama et al. 2016; Shigeta et al. 2017). Lipid extraction or mathematical corrections for mucus samples were not performed because glycoprotein and non-lipid components dominate the composition of mucus (Shephard 1994). After mucus sampling, dorsal muscle tissue was extracted from above the lateral section on the left side of same individual. Muscle tissue was also oven dried at 60 °C for 48 h, ground to a fine powder, and the effect of variable lipid content on $\delta^{13}C$ values corrected using the C:N ratio of each sample according to a fish general correction model by Post et al. (2007).

Stable isotope analysis was 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. Standards alanine [$\delta^{15}N$, 1.6 ± 0.2‰ (mean ± SD); $\delta^{13}C$, −19.6 ± 0.2‰ (mean ± SD)] and histidine [$\delta^{15}N$, −7.6 ± 0.2‰; $\delta^{13}C$, −10.7 ± 0.2‰] were used for calibration and quality control during the analysis through repeated measures. $\delta^{15}N$ and $\delta^{13}C$ were expressed as $\delta X = (R_{sample}/R_{standard}) - 1$, where X is ^{15}N or ^{13}C; R_{sample} is the $^{15}N/^{14}N$ or $^{13}C/^{12}C$ ratio of the measured samples; and $R_{standard}$ is the $^{15}N/^{14}N$ of atmospheric nitrogen or $^{13}C/^{12}C$ of Vienna Pee Dee Belemnite (VPDB). The analytical errors in the delta values were less than ± 0.3‰.

Data analysis and interpretation. All data analyses were conducted in R ver. 4.3.1 software. The Welch two-sample t-test, base::t.test() with unequal variance in R, was used to compare the means of biometrics between all males (n = 33) and females (n = 24) in this study. On the other hand, non-parametric tests were used to assess the differences in stable isotope ratios between individuals across the sampling period and this was done in two steps. First, the non-parametric Kruskal–Wallis test, base::kruskal.test() in R, was used to assess whether the observed trends in isotope ratios, with the interaction between sex and sampling month as an explanatory variable, were significant. If the Kruskal–Wallis test produced a significant result, then a multiple comparison test using the Steel–Dwass test was conducted using the NSM3::pSDCFlig() with "Monte Carlo" as a method in the function due to the relatively small sample size in this study. The advantage of using Steel–Dwass test than conventional methods, such as the Dunnet method, is its ability to solve

multiple comparison problems more easily (Takagi et al. 2003). When statistically comparing males and females, only datasets from the July catch ($n = 10$ males, $n = 10$ females) and August catch ($n = 9$ males, $n = 11$ females) were used due to sample size limitations. The sample sizes in the June catch ($n = 11$ males, $n = 1$ female) and September catch ($n = 3$ males, $n = 2$ females) were not large enough to perform statistical comparisons. Furthermore, a separate analysis was conducted for male individuals comparing the June catch ($n = 11$ males), July catch ($n = 10$ males), and August catch ($n = 9$ males) due to a sufficient sample size for statistical analysis.

The qualitative interpretation of multi-tissue isotope ratios was based on existing knowledge on $\delta^{15}N$ and $\delta^{13}C$. This approach was adopted due to the lack of specific turnover rates and trophic discrimination factors (TDFs) for $\delta^{15}N$ and $\delta^{13}C$ in Hasu fish tissues (fundamentally because this species' timid nature prevented them from smooth diet switch in our aquarium). We recognize the potential of the stable isotope clock, a method presented by Heady and Moore (2013), which takes advantage of the different turnover rates in two tissues to estimate the time since upstream migration (an analysis that would have been ideal for this study). Unfortunately, due to the unavailability of initial $\delta^{15}N$ and $\delta^{13}C$ for muscle and mucus tissues, along with the absence of specific turnover rates and TDFs, coupled with the inherently timid nature of the species, we were unable to directly estimate the time since upstream migration.

In closely related species, $\delta^{15}N$ and $\delta^{13}C$ tissue-specific turnover rates are larger in mucus tissue than in muscle tissue (Maruyama et al. 2016; Shigeta et al. 2017; Winter et al. 2019). Thus, when $\delta^{15}N$ and $\delta^{13}C$ of populations with many immigrant individuals were compared, mucus tissue should exhibit greater variation than muscle tissues. Whereas such trend is obvious only for some time after migration because faster mucus tissue can saturate faster than the slow-changing muscle tissue upon reaching isotopic equilibrium with the river environment after a long period of time, this saturation should not be observed in this study (half-life of mucus isotopic changes of three small cyprinid species were over two months; Shigeta et al. 2017). Theoretically, this variance is expected to indicate changes in the diet after movement of Hasu fish from Lake Biwa to Shiotsuo River, since riverine environments have higher $\delta^{13}C$ and lower $\delta^{15}N$ than lacustrine environments.

Results

Findings from the biometric measurements. No Hasu fish were caught in May despite our sampling efforts. In June, more male individuals were caught than female individuals (Fig. 1). In addition, the ratio of male to female Hasu individuals decreased with progression of the reproductive season.

On average, the standard lengths [220.0 ± 26.3 mm (mean ± SD) for all males and 187.5 ± 22.8 mm for all females] and wet weights were larger in males than in females in this study ($t = 4.98$ and 4.86, $d.f. = 53.94$ and 54.89, $p < 0.05$ in both tests). The gonad weight and gonado-somatic index were slightly larger in females than in males. However, only the gonado-somatic index was significantly different ($t = -3.34$, $d.f. = 39.52$ and $p < 0.05$).

In the June sampling, no food items were obtained from the guts of Hasu fish. However, the amount of gut content increased from July–August. The gut content analysis revealed that Ayu fish were the predominant food item of Hasu fish (Fig. 2). Other food items like small insect appendages (e.g., legs) and egg-like structures were also obtained. From June to September, all caught individuals had a Fulton's condition factor (K) value greater than one. The average K value decreased slightly in August before increasing again in September. It is also worth mentioning that there were no observable differences in the feeding frequency between males and females across the reproductive season.

Findings from the multi-tissue stable isotope ratio analysis. There were some observable trends in $\delta^{13}C$ and $\delta^{15}N$ for muscle and mucus tissues for both males and females across the reproductive season (Fig. 3). The slow-changing muscle tissue was relatively unchanged across the reproductive season, while the fast-changing mucus gradually approached isotopic equilibrium with the river environment. However, the fast-changing mucus never saturated. These trends in Hasu fish muscle and mucus tissues indicate a recent shift in diet from Lake Biwa to Shiotsuo River, suggesting that the rate at which individuals reach isotopic equilibrium varies among individuals.

In the June sampling, the $\delta^{13}C$ were higher in muscle tissues than in mucus tissues for both males ($n = 11$) and females ($n = 1$). From June to July, there was an increase in $\delta^{13}C$ for mucus in both males ($n = 10$) and females ($n = 10$), while $\delta^{13}C$ in muscle remained relatively unchanged. This higher $\delta^{13}C$ in July suggests that more individuals were approaching isotopic equilibrium of the river (which is higher than that of the lake). From the July to August catch, $\delta^{13}C$ for muscle tissue was not significantly different between (Kruskal–Wallis Chi-squared = 4.11, $d.f. = 3$, $p > 0.05$) males ($n = 9$) and females ($n = 11$) and $\delta^{13}C$ appeared to be saturated. However, there was a significant difference in mucus $\delta^{13}C$ (Kruskal–Wallis Chi-squared = 9.96, $d.f. = 3$, $p < 0.05$). Although the $\delta^{13}C$ for mucus tissue in females were lower than those in the previous month, and while those in males increased, the comparison test revealed that the differences were only significant between males caught in July and males caught in August (W *statistic* = 4.34, $p < 0.05$). When comparing males in the June, July

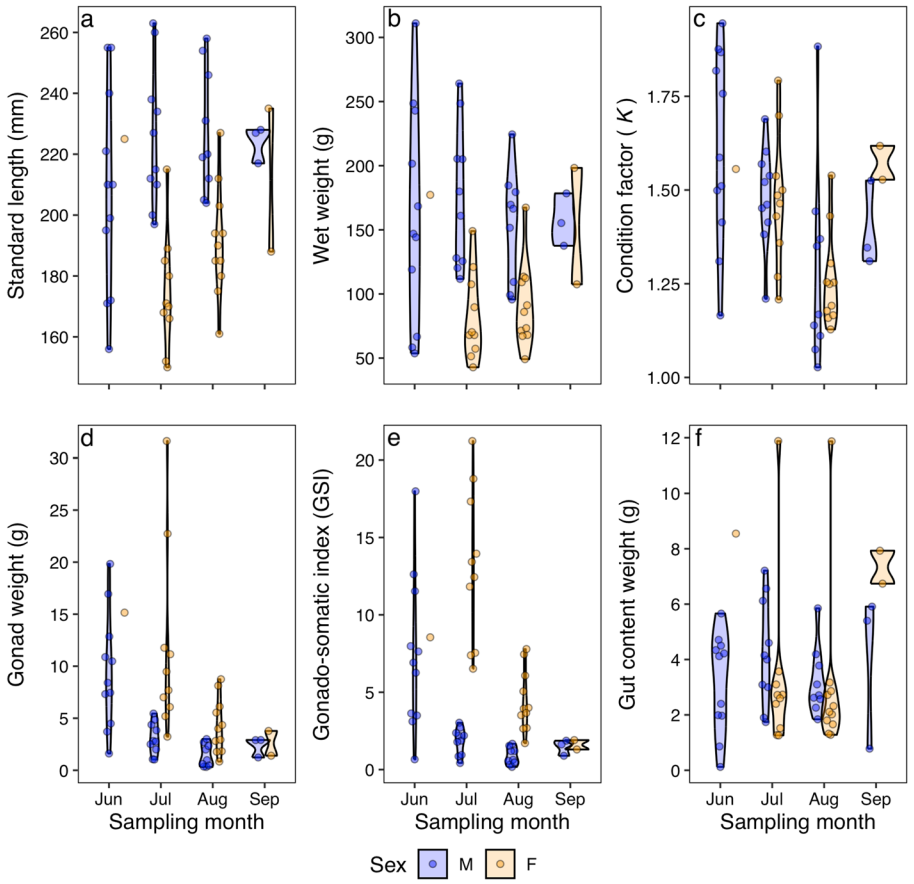

Fig. 1 Changes in biometric measurements of Hasu fish during the reproductive season in Shiotsuo River: standard length (**a**), wet weight (**b**), Fulton's condition factor (**c**), gonad weight (**d**), the gonado-somatic index (**e**), and gut content weight (**f**). The *blue* and *orange* violin plots (with points) indicate male and female Hasu fish, respectively. The violin plots indicate the distribution of each measurement, where the width of the plot at any given point indicates the density of the data. Violin plots and points were jittered to align with each other. In May, no fish were caught despite our sampling efforts; as a result, it was excluded from the plots

and August catch, the Kruskal–Wallis test also revealed significant differences in $\delta^{13}C$ of males caught in the 3 months (Kruskal–Wallis Chi-squared = 14.72, *d.f.* = 2, $p < 0.05$). However, the differences were only significant in $\delta^{13}C$ of individuals caught in July and those caught in August (*W statistic* = 3.33, $p < 0.05$). This also indicated that the males approached isotopic equilibrium with the river environment earlier than the females.

Similarly, the $\delta^{15}N$ values were higher in muscle than in mucus tissue of both males ($n = 11$) and females ($n = 1$) in the June sampling. In the June to July catch, there was no considerable difference from the previous sampling in $\delta^{15}N$ of muscle tissue in both males ($n = 10$) and females ($n = 10$) caught (Fig. 3). There was also more variation in $\delta^{15}N$ ratios for mucus in both sexes, suggesting that there was feeding on items with different isotope signatures. In the July to August catch, $\delta^{15}N$ values of mucus tissues were slightly lower in males ($n = 9$), while those in females ($n = 11$) were higher in comparison to the previous month (Kruskal–Wallis Chi-squared = 9.81, *d.f.* = 3, $p < 0.05$). The comparison test revealed that the differences were only significant between females caught in July and females caught in August (*W statistic* = 3.79, $p < 0.05$). The $\delta^{15}N$ values in muscle tissues were not significantly different between males and females (Kruskal–Wallis Chi-squared = 3.02, *d.f.* = 3, $p > 0.05$) and remained relatively unchanged across the sampling period, as is expected of slow turnover tissues. No significant differences were observed between the June catch, July catch, and August catch in the assessment of $\delta^{15}N$ in males (Kruskal–Wallis Chi-squared = 2.16 and 2.95 for mucus and muscle tissues, respectively, *d.f.* = 2, $p > 0.05$). From the catches, some individuals had $\delta^{15}N$ that were closer to the equilibrium of the lake than the river, indicating a recent change in diet from Lake Biwa to Shiotsuo River.

Discussion

New insights on the biometrics of Hasu fish and its feeding during reproductive migration. There was evidence of sexual dimorphism similar to a report by Tsunoda (2023). Males exhibited larger standard lengths and wet weights than females with each catch. Hasu individuals caught during this study were also larger (with average standard lengths of 220.0 ± 26.3 mm (mean ± SD) for all males and 187.5 ± 22.8 mm for all females caught in this study) than those reported by Tanaka (1964). This means that from 1964 to 2019, the length of reproducing Hasu male and female

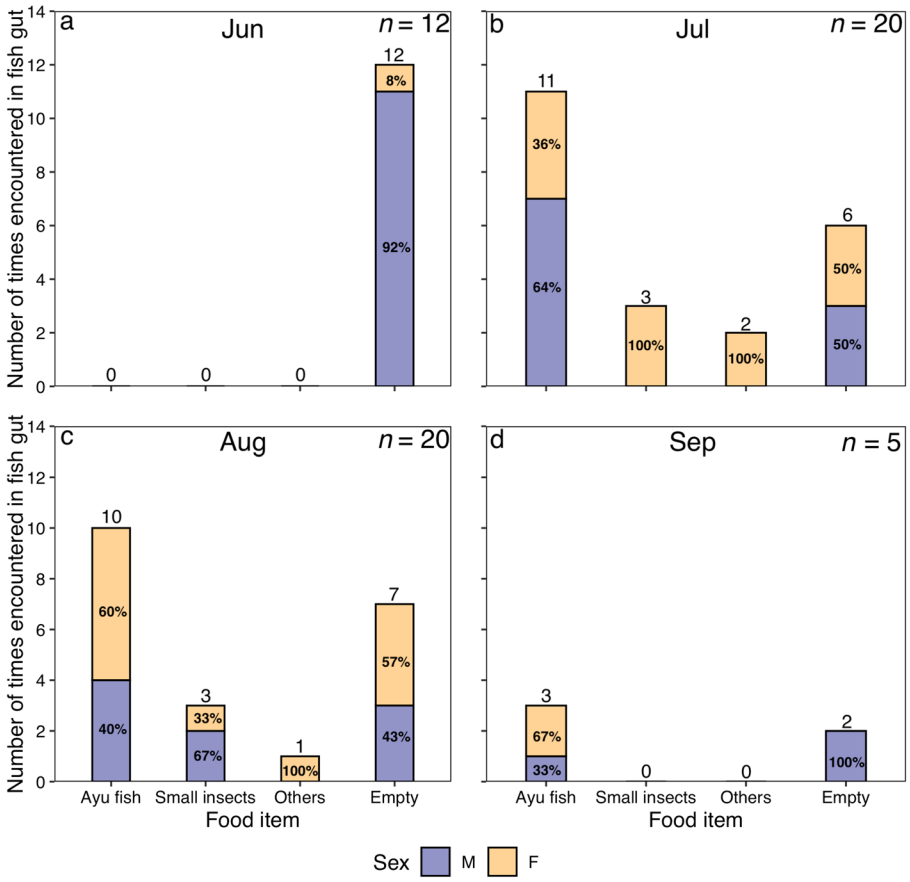

Fig. 2 The number of times food items were encountered in the guts of male (*blue*) and female (*orange*) Hasu fish caught in June (**a**), July (**b**), August (**c**), and September (**d**). In May, no fish were caught despite our sampling efforts; as a result, it was excluded from the plots. The numbers on top of each bar indicate the number of times a food item was encountered in the guts of Hasu fish, while the percentages (%) inside the bars indicate the proportion of male and female guts observed for each food item. The gut content of all fish were analyzed during each catch. In the plots, 'Others' included orange egg-like structures

individuals increased by approximately 37.5% and 44.2%, respectively. The increase in length may be attributed to a combination of factors, including improved fishery management practices, lack of competition for prey resources due to the decline in population size, and habitat restoration efforts that create more favorable conditions for fish growth (Tsunoda 2023). However, there is a need for focused research that incorporates the environmental factors in the Lake Biwa ecosystem to better identify which scenario is more likely. While males were larger in length and weight, females displayed slightly higher gonad weights and gonado-somatic indices, potentially reflecting greater reproductive investment (Gui and Zhou 2010).

Gut content analysis revealed, for the first time ever, the feeding behavior of Hasu fish during the reproductive season in the rivers. Ayu fish dominated the diet of Hasu fish, with limited feeding activity observed at the beginning of the reproductive season (Fig. 2). Hasu fish seem to allocate their energy resources strategically at the beginning of the reproductive season by temporarily reducing their feeding activity. This is probably to prioritize energy-demanding reproductive activities such as courtship, mating, and spawning (McBride et al. 2015).

All individuals caught during the reproductive season had a Fulton's condition factor (*K*) of greater than 1, indicating that all migrating individuals were healthy individuals (Mozsár et al. 2015; Fig. 1). The decrease in *K* value during the August catch could be a result of one of two likely scenarios. First, there could be a potential temporary decline in the overall health and condition of the fish population in the river. Several factors may contribute to this decline, including environmental changes, rising water temperatures, and potential alterations in food availability (Ficke et al. 2007). The subsequent increase in *K* value in September may indicate a recovery or a response to changing environmental conditions. This, in theory, explains why Hasu fish feed during the reproductive season. Secondly, it is also possible that the decline in *K* value was a result of new individuals approaching the study area. To further assess the likelihood of the second situation, $\delta^{13}C$ and $\delta^{15}N$ in Hasu muscle and mucus tissues could provide information about the differences in the timing of upstream migration among individuals. The next section discusses these variations.

Multi-tissue stable isotope ratio analysis reveals variation in the timing of feeding after upstream migration. Stable isotope ratio analysis of $\delta^{13}C$ and $\delta^{15}N$ revealed a recent change in diet in migrating Hasu individuals (Fig. 3). In the June sampling, both males and females exhibited higher $\delta^{13}C$ and $\delta^{15}N$ in muscle tissue, with slight fluctuations across the reproductive season, compared to mucus

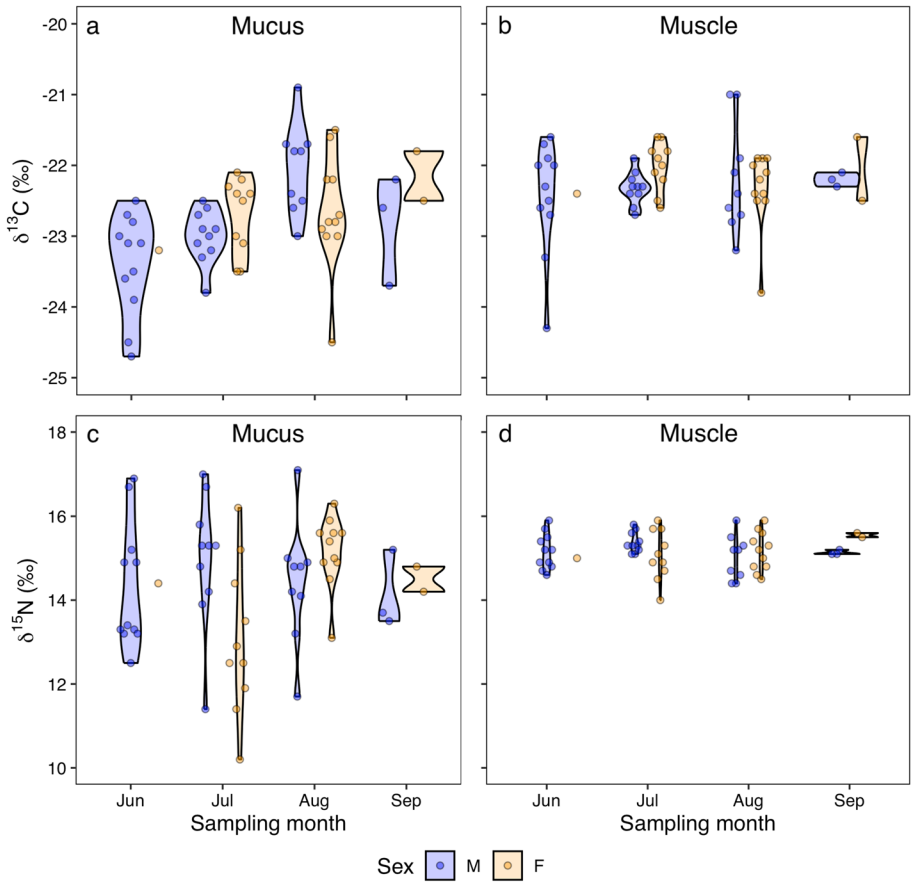

Fig. 3 Changes in carbon (**a**, **b**) and nitrogen (**c**, **d**) stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$, respectively) in mucus and muscle tissues of Hasu fish during the reproductive season in Shiotsuo River. The *blue* and *orange* violin plots (with points) indicate male and female Hasu fish, respectively. The violin plots indicate the distribution of stable isotope ratios, where the width of the plot at any given point indicates the density of the data. Violin plots and points were jittered to align with each other. In May, no fish were caught despite our sampling efforts; as a result, it was excluded from the plots. The change in $\delta^{13}C$ and $\delta^{15}N$ stable isotope ratios across the reproductive season reflects a change in Hasu fish diet from Lake Biwa to Shiotsuo River

tissue, which had distinct observable changes across the reproductive season. The slightly lower $\delta^{13}C$ in mucus tissue during the September catch may be attributed to changes in fish health. In our data, K decreased slightly in the August catch (Fig. 1). As such, the observed $\delta^{13}C$ in mucus tissue (during the September catch) could be a response to changing environmental conditions. The effects of changing environmental conditions on fish health and isotope incorporation rates were not assessed in this study, but we recommend further investigation into this relationship. Nevertheless, these observed changes, in $\delta^{13}C$ and $\delta^{15}N$, could also indicate a recent change in the diet of Hasu fish after migrating to Shiotsuo River, and perhaps the migration of Hasu individuals from Lake Biwa to Shiotsuo River.

Generally, Lake Biwa has lower $\delta^{13}C$ and higher $\delta^{15}N$ when compared to its tributaries (Yamada et al. 1998; Maruyama et al. 2001; Ito et al. 2015; Sawada et al. 2019). This is largely due to the differences in phytoplankton and periphyton and their reaction rates as primary producers. Thus, evidence of recent migration can be found in the gradual changes in the faster mucus tissue and little or no change in the slower muscle tissue. The $\delta^{13}C$ ratios gradually increased in mucus tissue, while the $\delta^{15}N$ ratios in mucus gradually decreased (especially after July) across the reproductive season. The little or no change in muscle $\delta^{13}C$ and $\delta^{15}N$, due to the slow turnover rate, further supports this recent diet switch and perhaps the recent migration of Hasu fish from Lake Biwa to Shiotsuo River.

Furthermore, the characteristic shifts in $\delta^{13}C$ and $\delta^{15}N$ of mucus tissue across the reproductive season suggest time lags and differences in the timing of feeding between males and females, with some males feeding earlier in Shiotsuo River (Fig. 3). This finding further cements the observations by Imamura (2018) during a study on the shorelines of Lake Biwa. By angling, Imamura (2018) observed that Hasu individuals were caught in an approximate ratio of 3 males per female, likely due to some early migrations of the males. Our stable isotope data also suggests that, even within the same sex group of Hasu fish, there are differences in the onset of feeding (and perhaps timing of upstream migration) in rivers. It is likely that $\delta^{13}C$ and $\delta^{15}N$ in the muscle and mucus tissues are similar between the individuals entering the river at different times (e.g., those in June and those in August). Based on the assumption that all migrating individuals are in equilibrium with their former environment and begin feeding on a consistent diet soon after upstream migration, then sampling at different times should potentially reveal early and late migrators.

In essence, if a Hasu fish caught in August has a similar $\delta^{13}C$ and $\delta^{15}N$ to a Hasu fish caught in June (with $\delta^{13}C$ and

$\delta^{15}N$ below the average of $\delta^{13}C$ and $\delta^{15}N$ in the August catch and is closer to $\delta^{13}C$ and $\delta^{15}N$ of the lake), then it can be deduced that the Hasu fish caught in August is a late migrator. On the other hand, if a Hasu fish is caught in August with an above average $\delta^{13}C$ and $\delta^{15}N$, closer to the $\delta^{13}C$ and $\delta^{15}N$ equilibrium of the river, then it can be deduced that the Hasu fish is an early migrator and has been in the river for some time. Early and late migrations have been documented in several species (Kynard and Horgan 2002; Quinn et al. 2007). Hasu fish, particularly the male individuals, are known to exhibit intraspecific aggression, especially toward other males in the same area (Hori 2022). Therefore, having both early and late migrations could represent an evolutionary response to mitigate competition among migrating Hasu fish.

In conclusion, this study found that the standard lengths of migrating Hasu fish have increased by over 37% since the 1960s despite the continued population decline for the past 70 years. For the first time ever, the study found that Hasu fish exhibit feeding behavior in the river, primarily on Ayu fish, during the reproductive season. This study also revealed that healthy mature individuals approached Shiotsuo River during the reproductive season, with males likely arriving earlier than females. $\delta^{13}C$ and $\delta^{15}N$ in muscle and mucus tissues also revealed variation in the onset of migration between individuals and between sexes, with males perhaps slightly migrating earlier than females. These crucial insights are fundamental to the management and conservation of Hasu fish and its ecosystem. As an apex predator and by feeding on important fishery species such as Ayu fish, we conclude that Hasu fish is important for maintaining the function and balance in the Lake Biwa ecosystem. Therefore, when setting fish traps in the rivers, the differences in the timing of upstream migration in Hasu fish need to be carefully considered. We recommend having perennial rivers accessible and free of barriers (e.g., dams) to the fish from June to September, when Hasu reproductive migration is most evident.

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Declarations

Conflict of interest We have no conflict of interest to declare.

Ethics approval All procedures complied with Japanese laws governing ethical conduct and the care and use of animals in research.

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PLOS ONE

RESEARCH ARTICLE

Using micro-CT to explore bone density variations in the skulls of the vulnerable *Opsariichthys uncirostris uncirostris* (Three-lips fish) during reproductive migration to a Lake Biwa tributary

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Abstract

Not much is known about the changes in bone density due to fish reproductive migration. We used micro-CT and inferential statistics to determine whether the relative bone density in the skulls of adult Three-lips fish, that seasonally upstream migrated to a Lake Biwa tributary, changed across their known reproductive season. The relative bone density significantly decreased as standard length and condition factor (*K*) increased in both sexes. This negative relationship is likely due to age and hormonal effects in the fish. Results from the bone density analysis also revealed that male Three-lips fish had potentially lower relative bone density (although not significantly different) than females during peak reproductive migration, i.e., July to August. On average, male Three-lips fish are larger in length and weight than females, and in many species, females prefer larger males to smaller males, viewing their size as an indicator of genetic fitness and their ability to provide protection. Resources in the skulls of Three-lips males may be distributed in such a way that increases reproductive success, i.e., size at the expense of quality. In addition, individuals with slightly less dense bones, particularly males, appeared later than those with denser bones during the peak of the reproductive season. The high energy demands involved with aggression in males, often requires resource mobilization from various tissue compartments and could explain the slightly lower density in the latter half of the peak migration. Furthermore, Three-lips individuals that migrated earlier and later during the reproductive season may have more energy reserves than those that had been in the river for some time, hence the variable bone density between individuals. This study serves as a foundation for future studies on the effects of migration, changes in physiology and age on bone density analysis of Three-lips fish and other species in various ecosystems.

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Introduction

Fish, such as the small teleost *Danio rerio* (zebrafish), which rely mostly on their skeletal architecture for structural support and mineral homeostasis, have been widely used to model skeletal morphogenesis in human skeletons due to similarities in developmental mechanisms [1, 2]. Although the skeletal system primarily informs function, feeding and mobility adaptations, the assessment of the fish skeletal system, such as bone density, can also offer a unique perspective in determining the intricacies of fish physiology, and provide insights into the health status of individuals [2]. The skeletogenesis of fish is progressive, often involving mineralization and compositional modulation of skeletal tissues [3]. Such a process reflects the interplay between environmental factors and endogenous physiological processes [4]. Integrating bone density analysis in the study of the potamodromous fish has potential to unveil a unique dimension linked to the reproductive migration of the species. Potamodromy, characterized by fish that complete their life cycles within freshwater systems, often involves extensive upstream migrations for spawning purposes [5, 6].

The relationship between bone density and reproductive migration has ecological significance. Changes in bone density can offer insights into the energetic demands and physiological adaptations associated with migratory endeavors [7]. As potamodromous species navigate challenging aquatic environments during reproductive migrations, alterations in bone density may serve as indicators of the metabolic investments required for successful migration and subsequent reproduction [2, 7]. A number of tools can be used to evaluate this relationship including, biochemical markers (e.g., C-terminal telopeptide), nutritional analyses, hormonal analyses and computed tomography [8]. Selecting the right tools for evaluation not only enhances our comprehension of the life history strategies of potamodromous fish but also provides a valuable tool for assessing the impact of anthropogenic activities on critical migratory corridors, ultimately contributing to the conservation and sustainable management of these essential fish populations [3, 4]. Understanding of bone density variations in fish, especially between sexes, can also provide insights into species-specific adaptations related to reproductive behavior [9]. The environment and dietary factors can affect nutrient recruitment in tissues, such as bones, and have an impact on the overall health of the fish [3].

Conventional health measurements like Fulton's condition constant (K), even though widely used, can not adequately identify drastic changes in fish energetics especially during the reproductive season when weight changes can be observed due to reproductive behavior [10, 11]. By assessing bone density, however, it should be possible to identify sudden changes in the health or energetics of migrating populations [12]. The newly developed technique micro-focus computed tomography (micro-CT), coupled with calcium hydroxyapatite (CaHA) phantoms, provides a powerful tool for studying bone density in fish [13]. Unlike conventional methods of assessing bone density, e.g., chemical methods that may alter the structure and composition of bones, micro-CT provides high-resolution three-dimensional imaging of bone structures, allowing for detailed quantitative analysis of parameters without altering the structure or composition of the bones [14–16]. The inclusion of CaHA phantoms in micro-CT protocols promotes standardization by mimicking the x-ray attenuation properties of bones, therefore enabling calibration of CT images to bone density [17]. This calibration is crucial for accurate and reliable measurements of bone density [13, 17]. The non-destructive nature of micro-CT also allows us to examine precious specimens and make informed decisions on the status of internal structures with minimal damage to the specimen [18].

Opsariichthys uncirostris uncirostris (Three-lips fish), an endemic yet vulnerable potamodromous fish that relies on lake–river migration for its reproductive migration in the Lake Biwa ecosystem, migrates and initiates feeding at different times in Lake Biwa tributaries [19].

Three-lips fish and its related species in Asia, which lack dental teeth, have evolved unique jaws to catch fish within the constraints of the cyprinid family [20]. Although the cost of migration has been discussed in evolutionary ecology of migratory fishes, not much is known about the changes in bone density related to reproductive migration. Therefore, this research aimed at exploring the bone density variations in the skulls of the vulnerable potamodromous Three-lips fish by using micro-CT derived bone density. The research questions for this study were as follows: is the bone density of the cranium in Three-lips fish related to size and health of the fish? Are there differences in the bone density of the carnium in Three-lips individuals sampled in different months during their reproductive season? Are there bone density differences in the cranium of males and females during their reproductive migration?

Materials and methods

Description of study site

The samples in this study were collected in the lower reaches of the Shiotsuo River (under the control of the Shiga Prefectural local government), which flows into Lake Biwa from the north. The river has a total length of 9 km and a basin area spanning 21.8 m^2 [21]. The river flows through mountainous terrain (95.8% of the total river catchment area), contributing to a steep gradient of 9.5 m/km, making it one of Lake Biwa's steepest rivers and a preferred reproductive upstream migration route for Three-lips fish when they seasonally migrate from May to September. Reproductive migration of Three-lips fish in Lake Biwa has been documented in several studies using conventional and environmental DNA (eDNA) analysis [22, 23]. A study on $\delta^{13}C$ and $\delta^{15}N$ stable isotopes in muscle and mucus tissues of Three-lips fish during their reproductive migration to the Shiotsuo River also indicates that the fish may migrate and inhabit spawning sites at varying times, with some individuals staying longer than others in the river [19]. Due to its steep gradient, the river is fast-flowing, and it is also characterized by gravel bottom substrates, all of which have been identified to be key drivers of Three-lips reproductive migration [22]. In addition to the ease of sample collection, Shiotsuo River is perennial, and there are no high weirs in the middle and lower reaches of the river, allowing for natural upstream migration of Three-lips fish. These characteristics make Shiotsuo river a conducive environment for conducting bone density studies on Three-lips fish during its reproductive upstream migration.

It is expected that Three-lips fish caught at different times during their reproductive migration in Shiotsuo River will have varying bone density in their cranium due to size and health, time the species was caught as well as sex of the individual.

Fish sampling and biometric measurements

Three-lips fish samples were collected monthly in the lower reach (2–3 km from the river mouth) of Shiotsuo River in 2019 during their reproductive migration from May to September using cast nets. Fish sampling in the Shiotsuo River was done with approval from the Department of Fisheries of Shiga Prefecture (permit number: 30–34, flag numbers: 274, 454 and 537). The sampling plan was systematic and a target of 20 Three-lips individuals was set for each month (i.e., 20 individuals over 5 months, in total of 100). Despite sampling efforts, only 57 individuals were collected from the planned 100 (Table 1).

The fish were sacrificed by placing them in ice water and in compliance with Japanese laws and standards. Since the fish were caught during their reproductive migration, no control specimens were obtained. Wet weight (g) and standard length (mm) were measured (to the nearest 0.01 g or 0.1 mm) in the field. The sex of the fish was confirmed in the field by dissecting the fish and checking the gonads for sperm and eggs. Fish were put in individual Ziplock

Table 1. Number of Three-lips fish individuals (males and females) collected in Shiotsuo River each sampling month.

Sampling Month	Number of Three-lips individuals collected		Sex ratio (males to females)
	Males	Females	
May	0	0	N/A
June	11	1	11:1
July	10	10	1:1
August	9	11	9:11
Seprember	3	2	3:2

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bags and kept on ice in a cooler box while in the field. After the field sampling, samples were transported to the laboratory at Ryukoku University and kept frozen below –22.5˚C until analysis. The Fulton's condition constant (K) was calculated for each fish according to the following formula:

$$K = 100 \times \frac{W}{L^3}$$

Where: W (g) is the wet weight of the fish and L (cm) is the standard length of the fish.

Sample preparation and micro-computed tomography

Prior to micro-CT scanning, the samples were placed in 70% ethanol for at least 48 hrs. to facilitate water removal. Next, the samples were cut along the dorsoventral axis just before the pelvic fin. This was done to ensure that the samples fit into a scanning container and field of view of the micro-CT scanner. The samples were then washed in absolute ethanol to remove any impurities on the surface of the fish and fixed in the scanning container together with a Micro-CT-HA phantom (QRM, Möhrendorf, Germany) containing 5 rods of Calcium hydroxyapatite (CaHA) with known densities (0, 50, 200, 800, 1200 mg CaHA/cm^3, respectively). The phantom was secured to the sample by means of Sellotape® (Fig 1A). All samples were scanned at 70 kV and 40 μA with a 1024 × 1024 resolution and YZ smoothing using an inspeXio SMX-100CT Micro Focus X-Ray CT System (Shimadzu Corporation, Kyoto, Japan). The slice size and voxel size were 0.079 ± 0.009 (mean ± SD) and 0.041 ± 0.043 (mean ± SD), respectively. The scan files were then exported as 8-bit bitmap files (Fig 1B) for subsequent processing in 3D slicer (a 3D imaging freeware) [24].

Measuring relative bone density in skulls of Three-lips fish

The cranium (skull bones) in scans were segmented (until the preopercle) using Otsu Thresholding (pixel intensity thresholding range: 60–255) due to its simplicity and speed in the Segmentation editor module of 3D slicer (Fig 1C–1E, [25]). The average pixel intensity, i.e., the specific area of the scan that has an accompanying value of x-ray absorption strength (on a grayscale, weak absorption is dark (black or 0 grayscale value) and strong absorption is bright (white or 255 grayscale value); [26]), for the segmentation on the cranium, superimposed on the original scan, was then recorded using the Statistics function of the Segmentation module of 3D slicer (Fig 1F). Similary, the average pixel intensities for each of the 5 phantom rods were obtained and used to create calibration curves (in the slope-intercept form) for converting pixel intensity of the scans to bone density (mg CaHA/cm^3). The R^2 value in all phantom calibrations was 0.99 (S1 Fig).

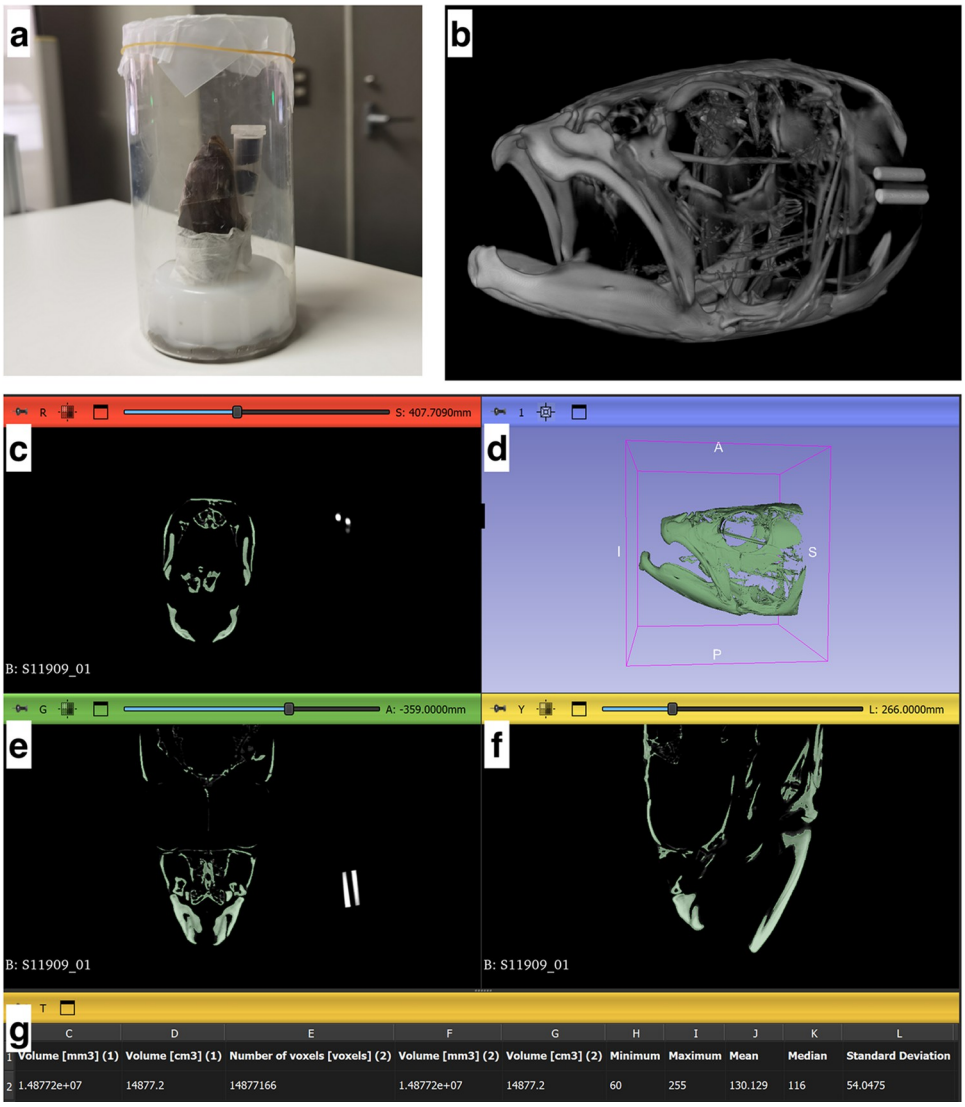

Fig 1. Workflow for assessing relative bone density using micro-CT. (a) The sample is placed in a scanning container with CaHA phantoms. (b) Scanned images are exported as 8-bit bitmap files for processing in 3D Slicer. (c–f) Bones are segmented using Otsu thresholding (pixel intensity range 60–255) and unwanted regions are removed using the scissors tool in 3D Slicer. (g) The pixel intensity in the segmented area, superimposed on the original scan, is recorded using the statistics function in 3D Slicer.

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Data analysis and interpretation

All data analyses were conducted in R ver. 4.3.1 software [27]. The relationship between the biometrics standard length (mm), sex and condition factor (*K*), as explanatory variables, and relative bone density (as a response variable) were assessed using a generalized linear model (GLM) with a guassian family [base::glm() in R]. Variable selection for the final model was then achieved through forward and reverse stepAIC [base::stepAIC() in R].

The asessment of bone density trends across the sampling period was done in two steps. First, the non-parametric Kruskal-Wallis test [base::kruskal.test() in R] was used to assess whether the observed relative bone density trends, with the interaction between sex and sampling month as an explanatory variable, were significant. If the Kruskal-Wallis test was significant, then a multiple comparison test using the Steel-Dwass test was conducted using the

NSM3::pSDCFlig() with "Monte Carlo" as a method in the function (due to the relatively small sample size in this study). The advantage of using the Steel-Dwass test over conventional methods, such as the Dunnet method, is in its ability to solve multiple comparison problems more easily [28]. When statistically comparing the relative bone density between males and females of Three-lips fish, only datasets from the July catch (n = 10 males, n = 10 females) and August catch (n = 9 males, n = 11 females) were used due to sample size limitations. The sample sizes in the June catch (n = 11 males, n = 1 female) and September catch (n = 3 males, n = 2 females) were not large enough to perform statistical comparisons. In parallel, the analysis was done on male individuals only comparing the June catch (n = 11 males), July catch (n = 10 males), and August catch (n = 9 males) due to a sufficient sample size for statistical analysis.

Results

Relationship between biometrics and micro-CT obtained bone density

In both males and female Three-lips fish, bone density decreased with increasing standard length and condition factor (K) (Fig 2A and 2B). The GLM model with gaussian family and stepAIC revealed that the relative bone density decreased as the explanatory variables standard length, condition factor increased and a tendency for lower bone density in males when compared to females (Table 2). The standard length and condition factor (K) had significant effects on the relative bone density in the GLM ($p < 0.05$) while sex did not have a significant effect on the relative bone density ($p > 0.05$). The negative relationship of condition factor and bone density was contrary to the expectation that healthier individuals, regardless of size, would have a larger bone density than slightly less healthier individuals. It is worth mentioning that the changes in standard length and condition factor (K) of Three-lips fish across different months have already been documented by Mvula and Maruyama [19]. In their paper, both the standard length and condition factor decreased during peak reproductive migration (July to August), which was attributed to environmental changes, rising water temperatures, and potential alterations in food availability. To effectively and statistically assess the effects of monthly variations in standard length and condition factor (K) on bone density, a larger sample size than the one currently obtained for this study is required.

Bone density distribution across the reproductive season

There was a significant difference in bone density between the July and August catches (Kruskal-Wallis $X^2 = 12.34$, $d.f. = 3$, $p < 0.05$). Bone density was lower in the August catch when compared to the July catch for both males and females (Fig 3). Although bone density was higher in females than in males between the July and August catches, pairwise comparison revelead that the differences were only significant between females caught in July and males caught in August ($W\ statistic = 4.39$, $p < 0.05$). In males only, the bone density was lower for each sampling month when compared to its previous month except in September when the bone density was higher than the previous month. However, the Kruskal-Wallis test revealed that the observed differences in relative bone density were not significantly different from each other. These results indicate that there may be differences in bone density due to sex, however, a bigger sample size is need to confirm this observation.

Discussion

Effect of age and environment on bone density

In this study, the relative bone density in Three-lips fish decreased as standard length and condition factor increased in both sexes (Fig 2, Table 2). As species grow, the rate at which

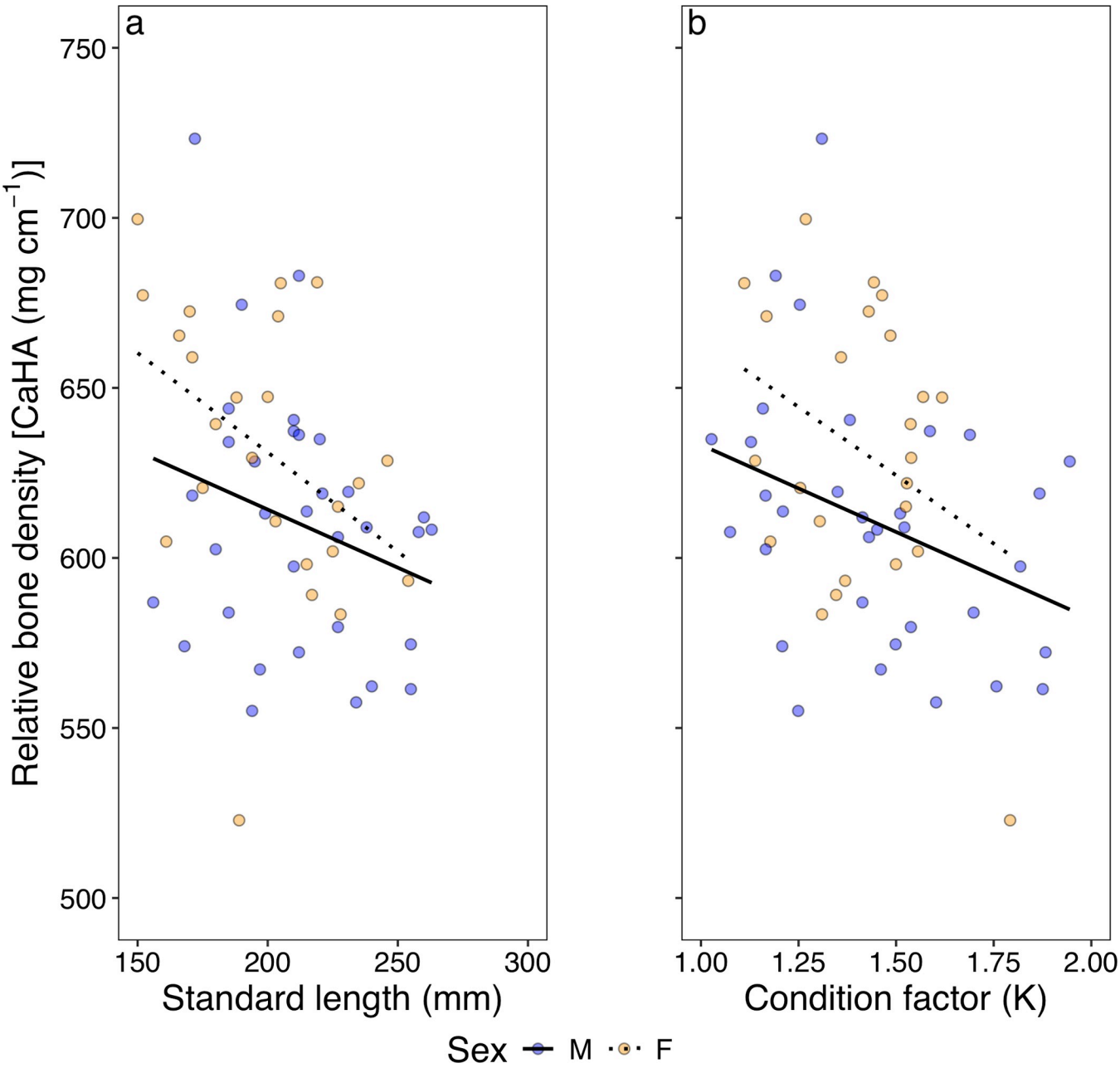

Fig 2. Relationship between relative bone density and (a) standard length and (b) condition factor. Blue and orange points represent male and female Three-lips fish, respectively. Black solid and dotted lines indicate the linear fit for males and females, respectively.

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materials, especially calcium hydroxyapatite, replenish in bones decreases probably due to age and hormonal effects in the fish [13, 29]. However, in the case of Three-lips fish, it is highly likely that the observed decline is due to hormonal effects, since migrating individuals during the reproductive seasonfall within the same older age group [30–32]. Fish bones play an important role in resource mobilization by acting as reserves for important nutrients. These nutrients, e.g., calcium hydroxyapatite, lipids, and proteins, are not only important for structural integrity of the skeletal system but may also act as an energy source for the fish in a stressed environment [2, 13]. Larger individuals require more energy to move their large

Table 2. Coefficients on relative bone density evaluated in relation to biometrics (standard length, condition factor and sex) as explanatory variables using general linear models (GLMs) with a gaussian family selected via stepAIC.

Explanatory variables	Coefficients (estimates ± standard errors)		
(Intercept)	778.56	±	42.20***
Standard length (mm)	−0.38	±	0.17*
Condition factor (*K*)	−50.23	±	21.63*
Sex[a]	−14.47	±	9.88

Significance levels
(***$p < 0.001$
**$p < 0.01$
*$p < 0.05$).

[a] The estimate for "Sex" in the linear regression model reflects the effect on the dependent variable specifically for males compared to females.

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bodies [33]. This could explain the slightly lower bone density in larger individuals. In addition, one would expect healthier individuals to exhibit higher densities compared to less healthier individuals. Even though all migrating individuals were healthy ($K > 1$), the negative relationship between condition factor (K) and bone density suggests some sort of resource investment strategy by Three-lips fish during their reproductive migration, perhaps to other tissue such as gonads which play a more direct role in spawning [34, 35]. Further research is required to ascertain resource utilization strategies from various tissues in Three-lips fish.

Bone density distribution across the Three-lips migrating period

Assessing the Three-lips fish bone density distribution across different months during their reproductive migration helps identify potential variations in bone density that may be influenced by seasonal changes or migration patterns. In particular, the higher bone density in June and September when fewer individuals are migrating could be a result of one of three scenarios: lack of competition between individuals, early and late migrations within the Three-lips fish population, or a response to strenuous reproductive activities. First, using stable isotope analysis and catch data, Mvula and Maruyama [19] demonstrated tentative differences in the timing of upstream migration from Lake Biwa to Shiotsuo River with potential early and late migrations within the population. It is possible that due to fewer fish in the river, there is reduced competition for mates and consequently the absence of high energy demanding activities like chasing other individuals [36, 37]. Alternatively, it is possible that the higher bone density in June and September is because of new individuals entering the reproductive migration sites. There are possible differences in the onset of feeding and consequently upstream migration between individuals [19]. It is likely that these individuals, during the early and late migration waves, have enough energy reserves in tissues other than bones to undertake upstream reproductive migration. As such, no drastic changes in bone density are expected. Finally, the higher bone density could be due to bone remodeling after strenuous reproductive activity (e.g., the upstream swim and spawning). According to Wolff's Law on bone remodeling, the structure (and consequently density) of bone tissue in healthy individuals will adjust in response to the mechanical forces and stresses applied to it [38].

Bone remodeling is a complex process that involves the conversion of mechanical signals into biochemical signals in cellular signaling [39]. There is evidence of mechanically induced bone remodeling in the jaws of teleost fish, such as cichlids [40]. As such, the mechanical demands of reproductive activities could induce remodeling response in the skulls of Three-

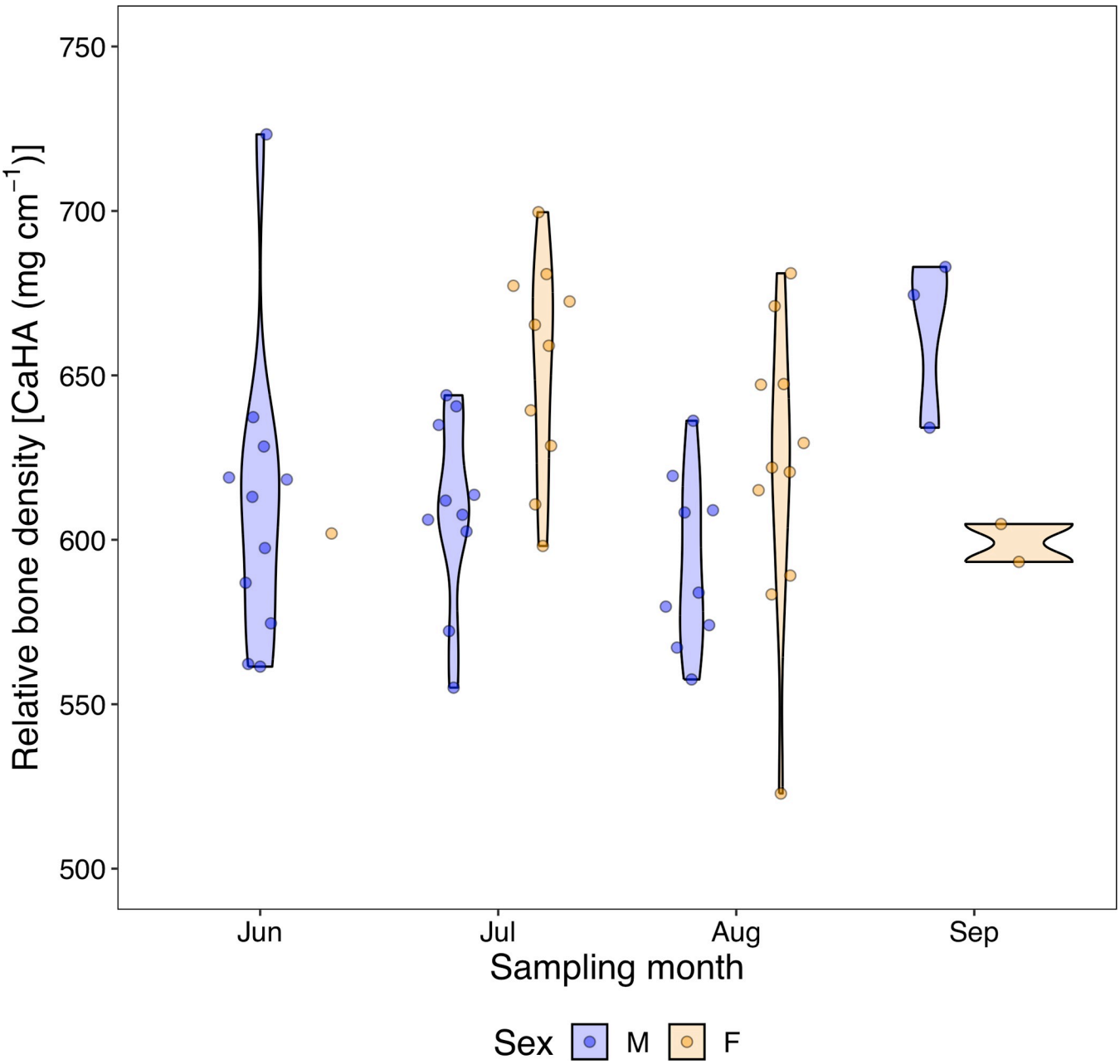

Fig 3. Relative bone density [CaHA (mg cm^{-1})] of Three-lips fish across the reproductive season. Blue and orange violin plots (with points) represent male and female Three-lips fish, respectively. The width of the violin plots indicates the distribution and density of the data. Data points and violin plots are jittered for alignment. May data is excluded due to no fish being caught despite sampling efforts.

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lips fish to cope with this stress. However, there is a need for further investigations on the behaviors of Three-lips fish before and after the peak migration period to draw conclusions on which scenario is more likely. For example, future studies may consider exploring the immediate impact of various stimuli (e.g., mechanical and chemical stimuli) on bone density in Three-lips fish. Furthermore, the bone density analysis in this study was done on the cranium as a whole, however, future studies should assess bone density differences in individual bones of the cranium. For example, these studies could focus on the differences in bone density

between the dentaries and premaxilla. This would not only provide insights into the dynamic nature of Three-lips fish bone remodeling in response to varying environments but could also inspire the development of new biotechnological approaches for bone tissue engineering and regeneration in humans, as is the case with zebrafish and other marine species [1, 41].

Sex roles potentially influence bone density in Three-lips fish

The results suggested differences between male and female Three-lips fish with males having a relatively lower bone density than females (Figs 2 and 3). Besides the hormonal differences between males and females, male Three-lips fish are, on average, larger in length and weight [30]. The effect of size and its role in reproduction might help explain the lower bone density in males than in females. A larger head could be a consequence of evolutionary processes that make males appear more threatening to other species, thus appealing to females during reproductive migration. Generally, females in many species prefer larger males, viewing their size as an indicator of their ability to provide protection [42–44]. In addition, large sizes may be an indicator of good health and genetic fitness in individuals [45]. Therefore, resources in the skulls of Three-lips males may be distributed in such a way that increases reproductive success, i.e., size at the expense of quality. In addition, individuals with slightly less dense bones, particularly males, appeared later than those with denser bones during the peak of the reproductive season (around July-August). Three-lips fish, especially males, are known to be aggressive, often chasing and biting other males in the same area [46]. Having denser bones during the peak of the reproductive season (around July-August), when more females are migrating, could provide a better reproductive advantage, not only as a weapon but also during resource mobilization. However, a larger sample size is required to confidently conclude on the effects of sex on bone density in Three-lips fish.

In conclusion, this study demonstrated variations in bone densities in Three-lips fish of different ages and health during their reproductive migration to a Lake Biwa tributary. Although not significantly different, the study also highlighted possible differences between male and female Three-lips fish. We recognize the limitations in this study and that the results must be treated with caution due to the relatively low sample size. Nevertheless, the findings from this study serve as a foundation for ecologists and biomechanics hoping to study the effects of migration, changes in physiology and age on bone density changes in Three-lips fish and other species in various ecosystems.

Supporting information

S1 Fig. Calibration curve for converting greyscale value to relative bone density using a MicroCT-HA phantom (QRM, Möhrendorf, Germany).
(TIF)

S1 File. Raw data underlying the findings in this study.
(CSV)

S2 File. R script underlying the analysis and figures in this study.
(PDF)

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