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Roles of male mate choice and its heterogeneity in reproductive isolation among mutually ornamented fishes

（オスによる配偶者選択と個体差が繁殖隔離に及ぼす影響：雌雄共通装飾をもつウグイでの検証）

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A Ph. D. Dissertation Submitted to

Division of Biosphere Science,

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Chapter 1

General introduction

Explaining why there are so many species existing in nature, is a heart of evolutionary biology. Understanding factors influencing the evolution of reproductive isolation is crucial to this goal because reproductive isolation maintains diversity by preventing the admixture of different species. Reproductive isolation involves many processes but can be largely classified into two categories: premating isolation and postmating isolation. Premating isolation is the mechanism that prevents mating with different species, usually through mating behavior: thus, premating isolation is often called behavioral isolation. Postmating isolation is the mechanism that prevents fertilization with heterospecific gametes or reduces the ecological/reproductive success of hybrids. Since premating isolation is not beneficial without postmating isolation, premating isolation is assumed to evolve no earlier than postmating isolation (Coyne and Orr 2004). Species pairs with postmating isolation but not premating isolation will suffer demographic damage by maladaptive interspecific matings (i.e. reproductive interference) (Gröning and Hochkirch 2008). Premating isolation, which reduces reproductive interference, is thus important in maintaining species diversity (Okamoto and Grether 2013).

Premating isolation is achieved by species-specific mating phenotypes, such as signals (e.g. ornamentation, vocal song) and reproductive timing/habitat, and mate preference for the species-specific mating phenotypes. It has been widely recognized that male mate preference is weaker than female preference, because the sexual difference in investment to offspring usually leads male-biased sex ratio, which intensifies the male-male competition and reduces the opportunity for male mate choice (Darwin 1871; Edward and Chapman

2011). Compared to females, male mate preference is less likely to evolve in response to indirect selection resulting from the increasing quality of offspring: the cost of intense male-male competition mitigates the indirect benefit of choosing better females (Härdling and Kokko 2005; Servedio and Lande 2006). Due to this dominant view of "indiscriminate males and choosy females" in terms of sexual selection within species, extensive efforts have been paid to uncover the roles of male mating traits and female preference for conspecific mates in premating isolation.

In theory, however, male preference for conspecific mates can evolve. In the mate choice comparing con- and heterospecific females, indirect selection may also be strong enough to shape male mate preference because hybrid fitness is often considerably lower than the fitness of pure crosses (Servedio 2007). Still, I know little about the role of male mate choice in reproductive isolation (but see Svensson et al. 2007; Roberts and Mendelson 2017). Mate preference is not uniform among individuals. Rather, mate preference can covary with behavioral tendencies throughout their life (Réale et al. 2007; Sih et al. 2015). A recent surge of interests in among-individual variation in behavior has led to massive descriptions about well-repeated individual behavioral variations and correlations among behavioral traits. However, the potential association between personality and behavioral isolation has surprisingly been underappreciated.

During my Ph.D., I conducted a series of studies on cyprinid fishes in Hokkaido, genus *Pseudaspius* (formerly described as *Tribolodon*)(Sakai et al. 2020) including *P. hakonensis* ("*Ugui*"), *P. sachalinensis* ("*Ezo-Ugui*") and *P. brandtii* ("*Maruta*") as a model system. These three species are deeply diverged (10–20 Mya: Imoto et al. 2013) but often hybridize (Sakai and Hamada 1985; Sakai et al. 2004). The three species are sympatry in the Northern Japanese archipelago and Sakhalin: *Ugui* widely range from Kyushu Island (Southern Japan) to Sakhalin Island and around the Sea of Japan and; *Ezo-Ugui* distribute from Northern Honshu Island (Northern Japan) to Sakhalin (Sakai et al. 2004; Watanabe et al. 2018); *Maruta* range along Sea of Japan from Toyama to Sakhalin Island and along Pacific from Kanagawa to Aomori (Sakai et al. 2004). They typically mature at age 2 and their lifespan is over 10 years (Sakai 1995, but see Atsumi and Koizumi 2017a). During the non-breeding season, the three species distribute across rivers and estuaries (Gritsenko 1982; Gavrenkov et al. 2004; Moriyama et al. 2008; Katano et al. 2010) and are hardly distinguishable in coloration (grey to silver color) and morphology (Nakamura 1969; Gavrenkov and Ivankov 1981; Ivankov et al. 2016). Throughout their life-history, they do not show territoriality and aggressiveness (Katano et al. 2010; Ogawa and Katano 2016). From spring to early-summer, matured fish aggregate to the middle reaches of rivers and form dense spawning schools (Ito 1975; Gritsenko 1982; Sakai 1995). Importantly, these species often form mixed-species spawning schools because they greatly overlap in breeding season and habitat (Gritsenko 1982; Sakai 1995). Spatiotemporal overlap in spawning allows hybridization among the three species but hybridization is generally limited in the wild. Hybrids are occasionally found in southern Hokkaido island (Northern Japan) (F1 hybrids, 0–4.8 %; later generation hybrids, 0–1 %: Sakai and Hamada 1985) but not reported in northern Honshu Island (Hanzawa et al. 1984; Sakai et al. 2007) and Sakhalin (Polyakova et al. 2015). These findings suggest that these species discriminate between conspecifics and heterospecifics and thereby avoid hybridization. Specifically, the nuptial coloration can be crucial to their premating isolation. The three species are morphologically similar, but both sexes exhibit species-specific conspicuous coloration only in their breeding season (i.e. mutual ornamentation, Figure 1) (Nakamura 1969). Thus, nuptial coloration in females might enable male mate preference for conspecifics.

In this thesis, I investigated the roles of male mate choice and its among-individual heterogeneity in reproductive isolation among *Pseudaspius* fishes. I specifically hypothesized that the nuptial coloration, which is common in both sexes but differentiates among species, assists mate preference for conspecifics. In chapter 2, I aimed to uncover the geographic pattern of nuptial coloration in *Ugui*, for the first step to investigate the role of nuptial coloration in reproductive isolation. *Ugui* is the most widespread species distributing both sympatric and allopatric regions with the other two species. Since nuptial coloration is a temporal trait only expressed during breeding season, it was quite difficult to document large-scale geographic patterns across Japan only by the researcher's field observations. Instead, I used the pictures on the internet, which was mainly uploaded by anglers across Japan. This study showed that the qualitative pattern of nuptial coloration is more diverse in allopatric regions than sympatry. Nuptial coloration pattern may be well conserved in sympatry potentially because selection against hybridization favors species-specific patterns in sympatry but not in allopatry. In chapter 3, I then experimentally investigated visual-based male species recognition using *Ugui* males and females of *Ugui* and *Ezo-Ugui*. Since females of the two species did not differ in their morphology and behavior, results strongly suggest that female nuptial coloration enables male species recognition. This study thus stressed that both male species recognition and female mating traits are important in reproductive isolation, which has long been underappreciated. In chapter 4, I found a positive correlation between risk-taking tendency in an emergence test and preference to heterospecifics in *Ezo-Ugui* males. This finding suggests that bolder males may prefer novel phenotypes or likely to take miss-choice for their mating partners. In chapter 5, I investigated postmating isolation among the three species by artificial breeding experiments. This study showed that the hatching rate of hybrid is as high as parental species. These series of studies suggest that male species recognition contributes to premating isolation and premating isolation plays a major role in maintaining species boundary among *Pseudaspius* species.

Chapter 2

Citizen science reveals geographic variation in the nuptial coloration of *Ugui*

Geographic variations in reproductive traits are important for evolutionary biology but often difficult to investigate because of the need for a large scale survey and the ephemeral nature of secondary sexual characteristics. Here, by using Web image searches (Google Images and Twitter), I revealed large scale geographic variations (> 1500 km) in breeding timing and nuptial coloration in the mutually ornamented fish, *Ugui*. As this fish is easily caught and many anglers upload their photos on the Web, I was able to find a total of 401 high-resolution photos from all over Japan. Breeding periods were determined from dates of photos with/without nuptial coloration, which matched with previous studies. I also found that breeding periods might have advanced three weeks over the last 80 years in the Chitose River, potentially due to climate change. Additionally, the pattern of latitudinal cline for breeding timing was revealed by delaying the timing in higher latitudes, although regional variations were also high. Finally, I quantified the patterns of nuptial coloration for the mutually ornamented fish, confirming that over 80 % of individuals showed typical colorations, but others showed rare phenotypes never before described, including an intermediate coloration between a potentially hybridizing species, *Maruta*. my web based method for estimating breeding phenology could be adopted for organisms with temporal sexual characteristics, such as plants (e.g. flowering time) and some fishes. Web image analyses are still preliminarily with many limitations, but could be promising for investigating the variations of visible traits.

Atsumi, K., & Koizumi, I. (2017). Web image search revealed large-scale variations in breeding season and nuptial coloration in a mutually ornamented fish, *Tribolodon hakonensis*. *Ecological research*, 32(4), 567-578. (best paper award)

Introduction

Massive amounts of information have been made available on the web by people worldwide, and the quantity of information is growing at an overwhelming rate. Scientists are aware of the importance and usefulness of information on the web. In particular, biologists have tried to actively acquire data from citizens via web-based projects (e.g., eBird); that is, researchers encourage citizens to send biological information to the researchers' websites or e-mail addresses. Such projects have successfully revealed phenology (Hurlbert and Liang 2012) and temporal changes in distribution (Taylor et al. 2014). However, in other research areas, such as linguistics and environmental science, researchers have been gathering information in an easier way by using internet search engines such as Google and Yahoo (Blair et al. 2002; Schade et al. 2013).

Three types of biological information can be obtained from web searches: search queries, videos, and photographs. These data often include dates and localities. Although the search query itself only represents citizen interest in the query, the frequency of searching for a certain organism could relate to the abundance of that organism, and temporal change in search frequency could represent temporal changes in biological phenomena (i.e., phenology). In fact, search query frequency has been used to detect flu epidemics (e.g., Ginsberg et al. 2009), range expansion, and phenology via Google Trends (Proulx et al. 2014). Videos and photographs contain biological information, and could be used in various ways: videos can be used to study animal behavior (Nelson and Fijn 2013; Yamazaki and Koizumi 2017); photographs have been used to detect invasive species (Miyazaki et al. 2016), color variations between (Allen et al. 2013; Sanz et al. 2013) and within species (Leighton et al. 2016), and for morphological study. These topics are crucial for ecological and evolutionary studies, and ecosystem management, but are often difficult to study because long-term or wide-range surveys are required. Because web-based citizen science can be cost-effective and well suited to macro-ecology (Kobori et al. 2016), web search-based research is becoming increasingly popular (Newman et al. 2012; Proulx et al. 2014; Leighton et al. 2016).

Ugui, a cyprinid fish, is a potentially good target for citizen science. Because they are easy to catch by fishing, many anglers upload pictures on the web. *Ugui* is distributed in Far East Asia, and ranges from Kyushu to Sakhalin and Sea of Japan (Sakai 1995). In this fish, males and females exhibit remarkable nuptial coloration during breeding season (e.g., Nakamura 1969). Current theories argue that such mutual ornamentation evolves via mutual sexual selection (i.e., both males and females are sexually selected) and/or social selection (i.e., ornamentation reflects status regardless of sex) (reviewed in Kraaijeveld et al. 2007). However, the dace shows little territoriality (Ogawa and Katano 2016) and high promiscuity (Ito 1975); thus, the mutual nuptial coloration is enigmatic. This nuptial color is known to intra-specifically vary, and may be affected by hybridization with similar heterospecific, *Maruta*, in Russia (Sviridov et al. 2003). In Japan, geographic variation in coloration is suggested (Nakamura 1969), and hybridization with *Maruta* is also reported (Hokkaido, northeastern Japan; Sakai and Hamada 1985), but there has been no detailed description of nuptial color variation. Understanding inter- or intra-population variation in nuptial coloration could be important for revealing the function of this ornamentation. Additionally, because this species ranges widely in latitude, breeding period varies geographically: February to March in Kyushu, southwestern Japan (Tabeta and Tsukahara 1964), and July in Hokkaido, northeastern Japan (Okada 1935). However, reports of breeding periods are relatively sparse.

Here, I investigated the variations in breeding season and nuptial coloration in *Ugui* using web image searches with specific analysis components. (1) First, I investigated the characteristics of photographs found from the web, such as locality and years, especially focusing on whether the frequency of photographs represent the relative abundance of the species (Ginsberg et al. 2009; Proulx et al. 2014). (2) Second, I estimated breeding periods based on the dates of photographs with/without nuptial coloration from three regions where breeding period has been reported. (3) Third, I evaluated latitudinal cline in breeding timing across Japan (ca. 1700 km), which is a basic but important macro-ecological pattern (O'Malley and Banks 2008; Kobayashi and Shimizu 2013). (4) Finally, I assessed inter- and intra-population variations in nuptial coloration, and considered allopatric and sympatric distributions with the potentially hybridizing species *Maruta*. To my knowledge, this is the first study to estimate breeding season from web image searches, which might be a promising approach for phenology studies.

Methods

Web search methods

I slightly altered the search methods for photographs based on analysis components (Table 1). Web search was conducted over a total of eight days in November 2016 (8–10, 14, 17–18, 20, and 27; see Table 1). (1) The first aim was to describe general characteristics of web photographs (e.g., frequency, locality, date). To find photographs from all over Japan with equal search effort among regions, I used the search query "*Ugui*", "*Haya*" (*Ugui* in the dialect of central and eastern Japan), or "*Ida*" (the dialect of western Japan) in Google Images. To find as many photographs as possible, I searched photographs every month from January 2008 to November 2016 by setting a custom date range (for example, I searched photographs uploaded from April to May 2008, then searched from May to June 2008). Exchangeable Image File Format (EXIF) data contain the date and time of photographs and camera settings, which are automatically recorded by the camera, and were used to investigate timing. If photographs did not contain EXIF data, photograph dates followed uploaders' descriptions. Photograph localities followed uploaders' descriptions, because none of the photographs contained localities in the EXIF data. Photographs without localities were omitted from the analyses. If uploaders did not describe the localities in detail, I defined their localities at the end point of the rivers described or the municipal office of the city, town, or village described. This should not significantly bias the results, because the potential maximum error (i.e., 0°35′ 27.60 in Sapporo city) is much smaller than the range I analyzed (i.e., 12°13′17.49 from Nobeoka, Kyushu to Teshio, Hokkaido). In addition, I only used only photographs with the date provided and visible nuptial coloration, because dace species in Japan can be easily distinguished by nuptial coloration. Species identification followed Amano and Sakai (2014) and the Japan Game Fish Association (2010); I omitted instances of nuptial coloration with broad black band and red cheeks (*Ezo-ugui* type) or one black and one red band (*Maruta* type, including two subspecies: *P. b. maruta*, which is distributed from Tokyo Bay to southern Iwate Prefecture; and *P. b. brandtii*, which is distributed from the Sea of Japan and Hokkaido; Sakai and Amano 2014) from analysis.

I also calculated the proportion of photographs that could be utilized for the analyses. I resampled photographs with nuptial-colored *Ugui*, and recorded the frequency of photographs for which the localities and dates were available. Here, I used "*Ugui*" as the search query and searched photographs every three month from 2014 to 2015. This search was conducted on 21 March 2017. Photographs found using this procedure were not used in the other analyses.

(2) To estimate local breeding periods, I compared the photograph dates with and without nuptial coloration (i.e., breeding and non-breeding status, respectively) within single rivers. I selected three rivers (Fig. 1a: Chikuma, Tama, and Chitose Rivers), because the estimated breeding periods were reported (Koyama and Nakamura 1955, Nakamura 1969 and Okada 1935, respectively) and enough photographs were available (> 30

photographs) in these rivers. To find more photographs, I searched via Twitter in addition to Google Images. Because the three rivers are located in eastern Japan, the search queries were "*Ugui*" and "*Haya*," which are the names of the dace in eastern Japan, combined with the name of the river (for example, "*Ugui* OR *Haya* Tama River"). Among the photographs without nuptial coloration, I selected the photographs that were clear enough to identify species from number of pre-dorsal scales or locality. In Tama and Chitose Rivers, because other similar species occur (*Maruta* and/or *Ezo-Ugui*), I discarded the matching photographs that had more than 36 pre-dorsal scales (which is not in the range of *Ugui*, but of *Maruta* and *Ezo-Ugui*) (Sakai and Amano 2014). As those two species are not distributed in Chikuma River, I did not select photographs based on scale number.

(3) To infer latitudinal cline, breeding timing was estimated from the dates of photographs with nuptial-colored *Ugui*. I used photographs from the above two procedures except for those without dates or nuptial coloration. In addition, to cover as many regions as possible, I searched specific regions where samples were sparse by adding the names of regions or rivers to the search query (e.g., "*Ugui*" OR "*Haya*" OR "*Ida*" "Osaka").

(4) To elucidate variation in nuptial coloration, I selected the photographs based on the above three procedures. Photographs without dates were included in the analysis, and photographs with clear nuptial coloration were selected; photographs taken in turbid waters with nuptial-colored *Ugui* were omitted from the analysis.

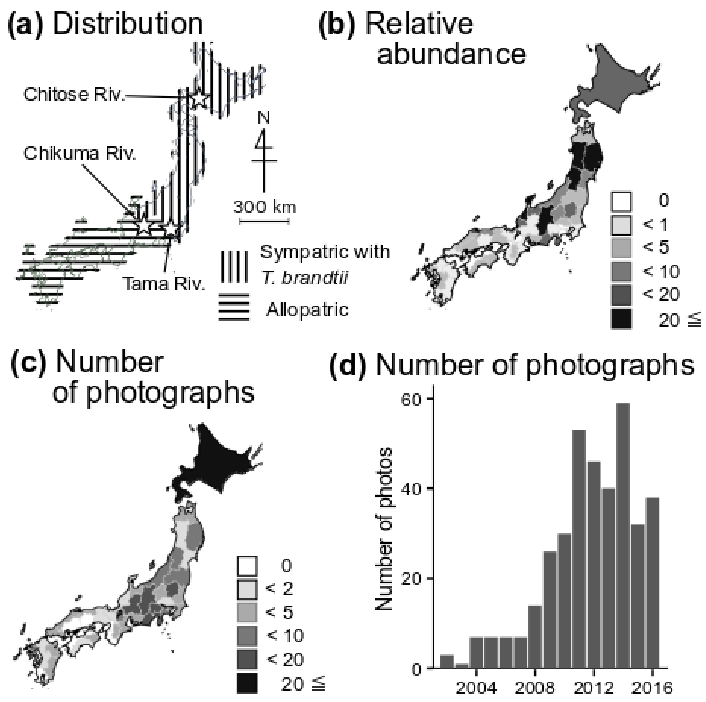

Fig. 1 **a** Distribution of the *Ugui*, *Pseudaspius hakonensis*, including sympatric and allopatric regions with a similar species with which it is potentially hybridizing, *Maruta* (*P. b. maruta* is distributed from Tokyo Bay to southern Iwate Prefecture, and *Maruta* is distributed from the Sea of Japan and Hokkaido: Sakai and Amano 2014). Stars indicate the three rivers where local breeding periods were estimated. **b** Relative abundances of *Ugui* calculated from the National Census on River Environments data. Numbers of photographs found for **c** each prefecture and **d** each year from 2002 to 2016

Characteristics of photographs from the web

I summarized photographs from the web based on prefecture, and analyzed the relationship between number of photographs and relative abundance of *Ugui*. Here, I hypothesized that photograph frequency is correlated with local abundance or biomass of the species. However, it is difficult to estimate local abundance or biomass in many places. Therefore, I used the relative abundance (i.e., proportion of the target species to the total species) from a large nationwide dataset (described below). Relative abundance is widely used as a good surrogate for abundance or biomass (Wen-Xiong Wang et al. 1995; Pearce and Ferrier 2001). I also considered the effect of human population size, because the number of photographs uploaded to the web should also correlate with human population size.

I used three datasets: (i) the number of photographs in each prefecture, (ii) relative abundance of *Ugui* in each prefecture calculated from National Census on River Environments data (data available at http://mizukoku.nilim.go.jp/ksnkankyo/03/index.htm; accessed 20 December 2016), and (iii) human population size in each prefecture in 2014 (data available at Statistics Japan, http://www.stat.go.jp/data/nihon/02.htm; accessed 27 December 2016).

In the river census dataset, I chose the most recent dataset available for each river, and did not use datasets from before 2010. Datasets were available for at least one river in every prefecture, with a maximum of five datasets used for a single prefecture. The census was conducted throughout each year (four seasons per year), and, because I used data from all seasons, the dace should be detected if present even though *Ugui* partially migrate. In each river, the proportion of *Ugui* to all fishes captured were calculated from the catch per unit effort standardized by the National River Census. The relative abundance in each prefecture was estimated by averaging the dace proportions calculated from the rivers surveyed in the prefecture. I only used the individuals identified as *Ugui* (*P. hakonensis*), and did not use unidentified individuals (i.e., *P. spp*.) which can contain other species of *Pseudaspius*.

I constructed a generalized linear model (GLM) with log link and Poisson error distribution. The number of photographs was used as a response variable, and the relative dace abundance and human population sizes were used as independent variables. All statistical analyses were performed using R (R Development Core Team 2016).

Local breeding period estimation

During breeding season, *Ugui* of both sexes show obvious nuptial coloration, which typically consists of a combination of three red/yellow and two black bands (Fig. 2b–c, Nakamura 1969). During non-breeding season, they show silver to greyish color with two inconspicuous grey bands (however, lowermost orange/red bands may occasionally exist; Nakamura 1969). Therefore, I assigned one of two scores to photographs: the dace showed obvious orange/red bands or patches, score 1; pale/no orange/red band or patches, score 0. When multiple individuals with different body colorations were in the same photographs, I separately scored those individuals. However, individuals showing the same coloration pattern within one photograph were treated as a single data point. Dates of photographs were transformed into the number of days from 1 January regardless of year (i.e., May 30 was transformed into 150); inter-year difference was not considered because of limited sample sizes.

As *Ugui* spawns from spring to early summer, the average scores of breeding color (presence = 1, absence = 0) should be lower from late summer to early spring, and higher in spring and early summer. Therefore, a quadratic relationship is expected between dates and color scores. Hence, I employed a logistic regression using the date and its quadratic term as explanatory variables. Here, the ranges of days for which regression lines predicted > 0.5 color score were defined as estimated breeding periods. To reduce potential scoring biases, a single person (KA) scored all the photographs, and consistency was confirmed by a blind test that used 50 random photographs (94% of scores were consistent). I also confirmed little observer bias with an additional blind test: 96% of scores judged by another person were consistent.

Latitudinal cline in breeding timing

As spawning of *Ugui* is strongly affected by water temperature (e.g., Tabeta and Tsukahara 1964; Nakamura 1969), I expected a latitudinal cline in breeding timing, which is usually found in many taxa (e.g., (O'Malley and Banks 2008; Kobayashi and Shimizu 2013). Therefore, I regressed the dates of photographs that showed the individuals with nuptial coloration against latitude. I assumed that the regression line represented the mean breeding timing at the given latitude. Although estimating local breeding period requires a relatively large dataset (see "Local breeding period estimation" in the Results), collecting many photographs from nearby areas should allow insight into the mean breeding timing around the region. Altitude may also affect regional temperature (Caissie 2006), but I did not consider altitude, because altitude can be sensitive to error caused by uncertain photograph localities. Longitude was not considered either, because latitude and longitude were strongly correlated ($r = 0.74$).

Variation in nuptial coloration

Detailed coloration was evaluated based on seven features: Rp1 and Rp2, red patches on the head located on the upper operculum and lower cheek, respectively; Rb1, Rb2, and Rb3, red bands on the body located above Bb1, on the lateral line, and below Bb2, respectively; and Bb1 and Bb2, black bands on the body located between Rb1 and Rb2, and Rb2 and Rb3, respectively (Fig. 2a). I subjectively evaluated the colors with identifiable criteria: I did not use color hue from compressed photographs (e.g., jpeg and png), because actual color hue in those images at pixel scale may significantly differ from what I perceive (Stevens et al. 2007). Coloration of each region was categorized into four ranks (red/orange, 2; pale red/yellow, 1; silver/grey, −0; black/dark grey, −1). To reduce potential scoring biases, a single person (KA) scored all the photographs, and consistency was confirmed from a blind test that used 210 points from 30 random photographs (92.4% of scores were consistent). Additionally, 80.5% of scores were consistent with those from another person. When multiple individuals with different body coloration were taken in the same photograph, I separately scored those individuals. However, individuals that showed the same coloration in one photograph were treated as a single data point.

To reduce the dimensions of the data, principal component analysis (PCA) was conducted. By checking the contribution of each trait to PCs, I can easily evaluate overall trait combination patterns. As the first two PCs accounted for most of the variance (75.9%), I used PC1 and PC2 values in subsequent analyses.

Geographical pattern in nuptial coloration was investigated. First, I examined correlation between PC values and latitude, because secondary sexual traits often show a latitudinal cline that is more elaborate in lower latitudes (Chui and Doucet 2009; Kawajiri et al. 2009). Second, I assessed a potential effect of hybridization with *Maruta* on nuptial coloration (Sviridov et al. 2003) by comparing the variance and mean values of PCs between sympatric and allopatric areas using the F-test and Welch's t-test, respectively. Sympatric and allopatric areas were categorized at the basin scale based on Sakai and Amano (2014). Body conditions and environmental factors may affect nuptial coloration (Morrongiello et al. 2010). However, because such information was rarely described by uploaders, I could not consider those factors.

Results

Characteristics of photographs from the web

Overall, I found a total of 401 photographs of *Ugui* from most areas of Japan (Table 1); of the 42 prefectures in which *Ugui* were found during the National River Census and the 46 prefectures that make up their known range, I acquired *Ugui* photographs from a total of 39 prefectures. I was able to utilize approximately half of the photographs from the web: among the 115 resampled photographs with nuptial coloration, both locality and date were available for 43.5% of the photographs. Among 401 photographs, 136 photographs (33.9%) contained dates, but no photograph contained localities in their EXIF data.

The number of photographs found during this analysis component (characteristics of photographs from the web) varied among regions, from 0 (nine prefectures in western Japan) to 52 (Tokyo) (Fig. 1c). GLM showed that both dace relative abundance and human population size positively affected the number of photographs ($P < 0.001$, Fig. 1b, Table 2). Most photographs dated from 2002 to 2016 (Fig. 1d), with a rapid increase from 2008 to 2011. I also analyzed relative abundance and phenology with Google Trends: the overall pattern showed peaks during early summer–early autumn, which might represent spawning season, but regional analysis was impossible because of small sample sizes (Online Resource Fig. S1C).

Local breeding period estimation

I found 33, 98, and 72 photographs taken in Chikuma, Tama, and Chitose Rivers, respectively. Regression curves were convex-shaped in Tama and Chitose Rivers (Fig. 3b–c). In Tama River, estimated range was almost the same at the ending, and two weeks earlier at the beginning of the previously reported breeding period (Nakamura 1969). In Chitose River, length of the breeding period was four times longer, and the peak of the curve was approximately three weeks prior to the peak of the breeding period reported 80 years ago (Okada 1935). In Chikuma River, the regression curve was sigmoid-shaped, and its peak was located outside of the known breeding period, which is due to the lack of data from January to March. However, the ranges of the dates of photographed nuptial-colored fish were well within the reported breeding periods (Fig. 3a).

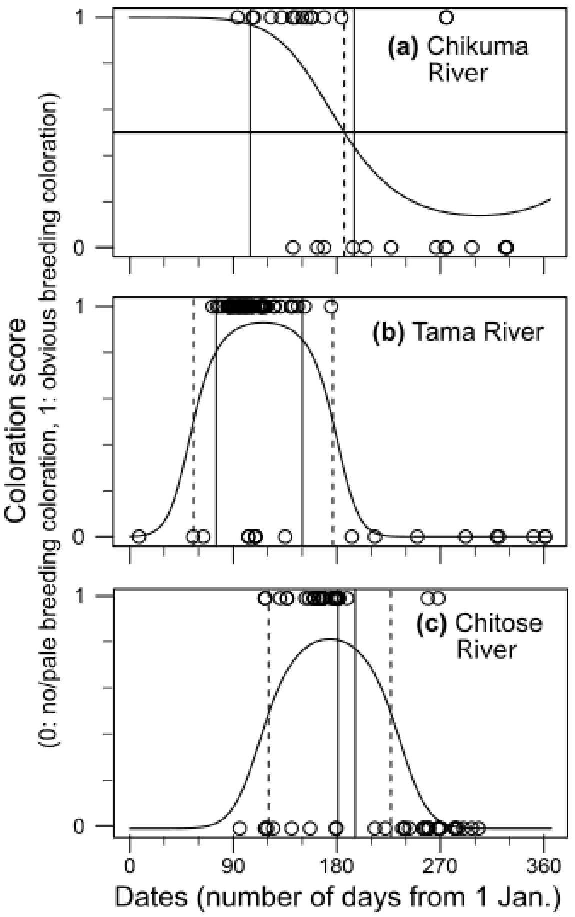

Fig. 3 Estimation of breeding periods in **a** Chikuma, **b** Tama, and **c** Chitose Rivers by quadratic logistic regression. Photograph dates were calculated taking 1 January as day 1 (i.e., 30 May was transformed into 150). Dashed lines indicate the day for which the regression curve predicted 0.5 breeding color value. Solid lines indicate the breeding periods reported in previous studies (Okada 1935; Koyama and Nakamura 1955; Nakamura 1969)

Latitudinal cline in breeding timing

I found a clear latitudinal cline in the dates of photographs that showed individuals with nuptial coloration across the Japanese archipelago (Fig. 4), which revealed that the mean breeding period was delayed in higher latitudes. This relationship was still significant after removing samples from Tama, Chikuma, and Chitose Rivers ($r = 0.27$, $P < 0.01$), where sample sizes were large. Estimated breeding timing was within the reported breeding periods except for a few cases (Fig. 4). Estimated breeding timing is mid-March in Kyushu, which is the southern margin of the distribution, and May in northern Hokkaido, northeastern Japan. I also found large variations in the dates when the dace showed nuptial coloration within the same latitude, which can indicate regional variation or long breeding periods within the same rivers. Additionally, five individuals showed nuptial coloration in late August and early September.

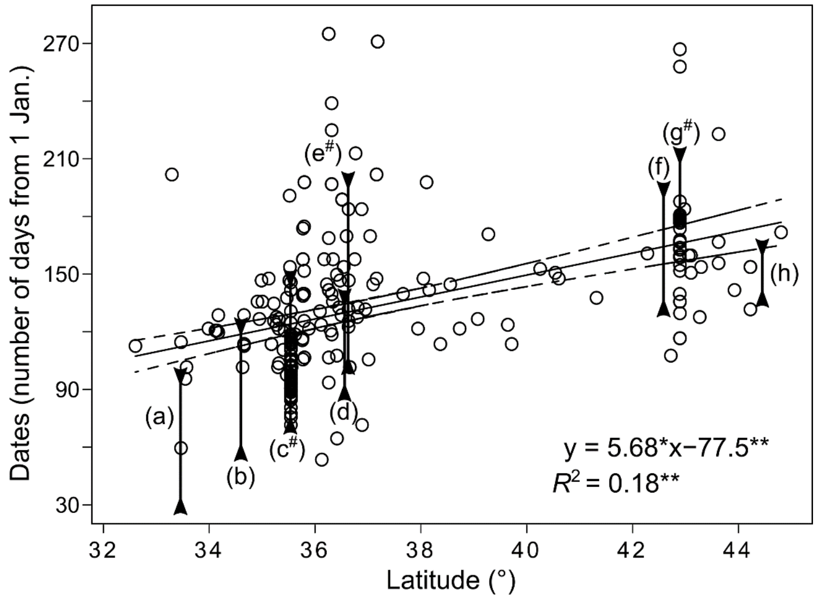

Fig. 4 Latitudinal cline in breeding timing of *Ugui* estimated from the dates of

photographs with nuptial coloration. Photograph dates were calculated taking 1 January as day 1 (i.e., 30 May was transformed into 150). Vertical lines indicate previously reported breeding periods in **a** Matsuura River (northern Kyushu, (Tabeta and Tsukahara 1964)); **b** Toba (central Honshu, (Ishizaki et al. 2010)); **c**[#] Tama River (Tokyo, see Fig. 1a, Nakamura 1969); **d** Tochigi Prefecture (central Honshu, (Iwamoto and Kanoki 1983)); **e**[#] Chikuma River (central Chubu, see Fig. 1a, (Koyama and Nakamura 1955)); **f** Mu River (central Hokkaido, Sakai 1995); **g**[#] Chitose River (central Hokkaido, see Fig. 1a, Okada 1935); and **h** Teshio River (northern Hokkaido, Ito 1975). [#]Rivers for which breeding periods were also estimated in this study (Fig. 3). *$P < 0.01$, **$P < 0.001$

Breeding color analyses

Of the total of 313 photographs, 80.8 % (253) showed the typical nuptial coloration reported in Nakamura (1969), with two red patches on the head (Rp1–2), three red (to yellow) bands (Rb1–3), and two black (to gray) bands (Bb1–2) (Fig. 2b–d). The other 60 individuals lacked some of the red patches/bands (i.e., showing silver/greyish color), but some general patterns (clusters) appeared. For example, 8.3 % (26) lacked Rb1–2, 5.8 % (18) lacked Rp1 and Rb1–2 (Fig. 2e), 3.5 % (11) only lacked Rb2, and less than 1 % lacked Rp2 and Rb3. Of the 11 individuals that lacked Rb2, three from Tama River had dark-grey Rb2 (Fig. 2h). Most individuals showed black to grey Bb1–2. However, in two individuals from central Honshu and northern Kyushu, Bb1–2 were orange/white and orange, respectively (Fig. 2f–g). Correlations in colorations of Rp2 to Rb3, Rb1 to Rb2, and Bb1 to Bb2 were strong ($r > 0.80$, $P < 0.001$ after Bonferroni correction), and those of Rp1–2 and Rb3 to Bb1–2 were significant but weak ($-0.29 < r < -0.22$, $P < 0.001$ after Bonferroni correction, see Fig. 2i). This means that (i) red patches/bands largely co-vary positively,

(ii) two black bands co-vary positively, and (iii) red patches/bands vary independently from black bands. I could not find orange/red spots within Bb1–2, which are frequently found in Primorye, Russia (Sviridov et al. 2003).

PC1–2 values were not significantly correlated with latitude ($r = -0.05$, $P = 0.44$ and $r = 0$, $P = 0.94$ for PC1 and PC2, respectively). Additionally, variance and average of PC1 values did not differ between allopatric and sympatric regions with *Maruta* (average, $t = -0.46$, $P = 0.64$, Welch's t-test; variance, $F = 1.30$, $P = 0.14$, F-test). However, PC2 showed greater variance in allopatric regions ($F = 0.44$, $P < 0.001$, F-test), mainly because of the two individuals that had orange Bb1, as mentioned above (Fig. 2j), whereas average did not differ between allopatric and sympatric regions ($t = -1.70$, $P = 0.09$, Welch's t-test). Variations within Chikuma, Tama, and Chitose Rivers were large and overlapping but not equal (Fig. 2j).

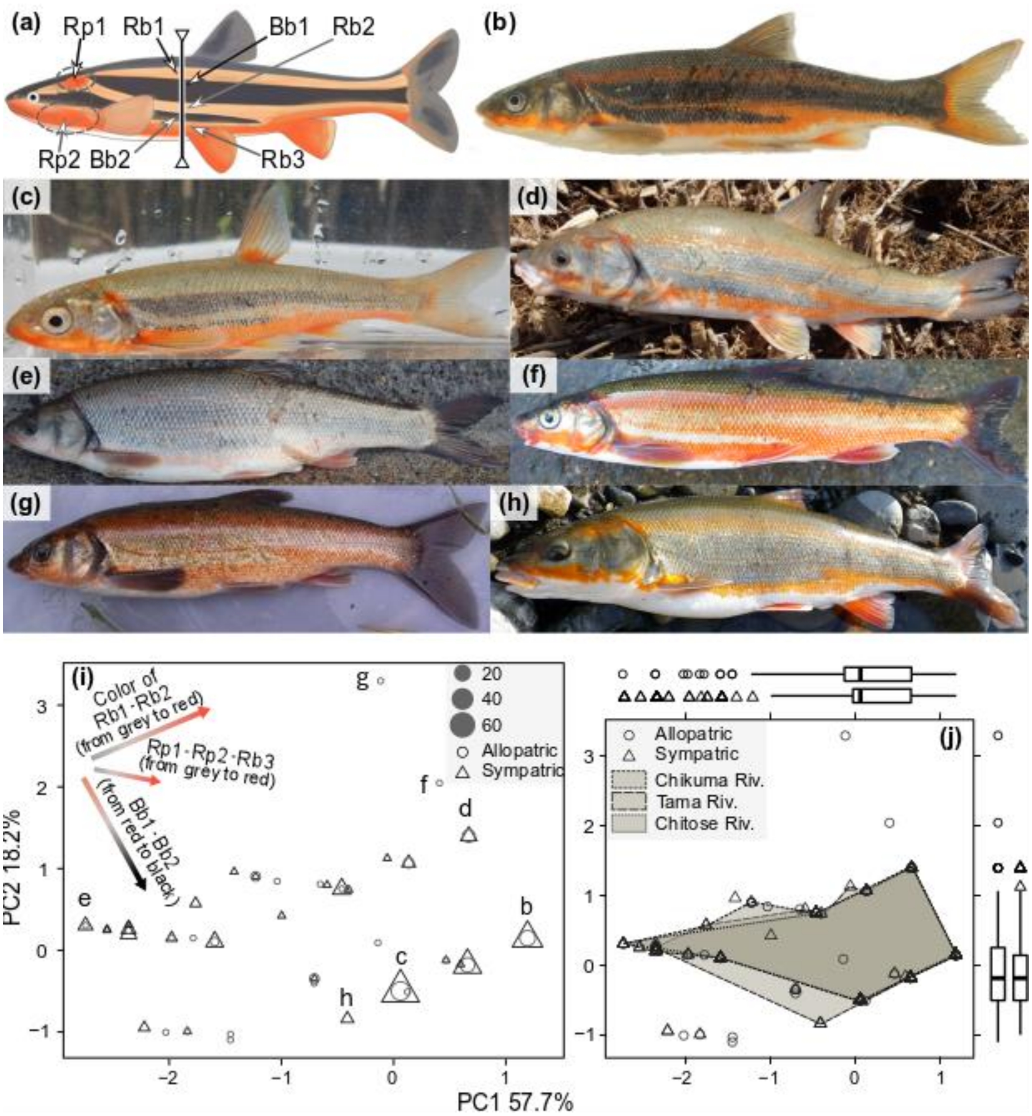

Fig. 2 **a** Schematic diagram of *Ugui* from seven regions used to evaluate nuptial coloration. **b**–**h** Some photograph examples of variations in nuptial coloration (**b**–**c**: typical coloration with difference in the color of Rb1–2): **b** orange/red Rp1–2 and Rb1–2, and black/dark grey Bb1–2 sampled in Sapporo, Hokkaido; **c** orange/red Rp1–2, yellow Rb1–2, orange/red Rb3, and black/dark grey Rb1–2 in Chiba, central Honshu; **d** orange/red Rp1–2 and Rb1–2, and grey Bb1–2 sampled in Bibi River, Hokkaido; **e** grey Rp1, pale red Rp2, grey Rb1–2, pale red Rb3, and grey Bb1–2 sampled in Akkeshi, Hokkaido; **f** orange/red Rp1–2 and Rb1–3, and white/orange Bb1–2 in Gifu, central Honshu; **g** orange/red Rp1–2, Rb1–3,

and Bb1–2 sampled in Fukuoka, Kyushu; and **h** orange/red Rp1–2 and Rb1, black/dark grey Rb2, orange/red Rb3, and black dark grey Bb1–2, which is an intermediate phenotype between *Pseudaspius hakonensis* and *Maruta maruta* sampled in Tama River, Tokyo. **i**–**j** PCA results; samples from allopatric and sympatric areas with *P. brandtiI am* shown as circles and triangles, respectively. **i** Sample sizes are represented by plot size, and color variations of each region are indicated by arrows. **j** Variations in allopatric/sympatric areas are represented as box plots, and variation ranges within rivers (Chikuma, Tama and Chitose) are shown. **b**–**h** were photographed by citizens or researchers, and printed with their permission

Discussion

Potential of web-based data for studying reproductive biology

I demonstrated the usefulness of web-based citizen data to investigate breeding season and the associated latitudinal cline, and nuptial color variations of a mutually ornamented fish, *Ugui*. It is now widely recognized that global climate change significantly affects organismal phenology, including breeding period (Walther et al. 2002). Web image searching can facilitate comparison between current and past phenologies in a wide range of species. In my case, the mean breeding period of *Ugui* in Chitose River was mid-July in the 1930s (Okada 1935), but was estimated to be mid-June based on photographs taken after 2009, which might be due to climate change or anthropogenic impacts.

Breeding period can directly influence population divergence via temporal reproductive isolation (Ketterson et al. 2015) and candidate genes for phenology, such as clock genes, are known to vary with latitude (O'Malley and Banks 2008). Empirical evidence on the latitudinal cline of breeding phenology, however, is limited. I successfully estimated the latitudinal pattern of breeding timing in *Ugui* over 1500 km, with only several days of web searching. Differences of 30–40 days in average breeding timing might contribute to intra-specific divergence. Future research can utilize this geographic variation to elucidate the temporal and spatial components of genetic variations within this species.

I also found large variation in nuptial coloration across Japan. The variation had some common trends, such as correlated changes within similar color patches/bands (i.e., red–yellow or black–gray) but not between the color patches/bands. Variation within populations was also large, although some geographic patterns still existed. Nuptial color variation has been occasionally mentioned in *Ugui* (Nakamura 1969; Sviridov et al. 2003), but this web-based approach is the first to quantitatively describe the variation in Japan.

Web-based data collection is useful for species that are frequently photographed by citizens and their photograph localities are easy to obtain (e.g., not conserved species). In particular, I was able to use web photographs with a relatively high proportion (i.e., 43.5%) that had available locality and date data compared with another similar study (26% in Leighton et al. 2016). This is probably because many photographs were uploaded on anglers' blogs, which provide accurate date and approximate localities (e.g., names of rivers, cities, or villages) in many cases. Phenological traits may be able to be estimated for species that (i) change body colorations or shapes (e.g., plants, cyprinid, and salmonid fishes) or (ii) migrate to spawning habitat during breeding seasons (e.g., migratory birds). In addition, variations in coloration could be revealed using citizen photographs, especially in organisms with discrete traits such as number of bands/spots, which can prevent observational errors. Although coloration and morphology seem easy to analyze using citizen photographs, various potential biases may exist because these characteristics are highly sensitive for to photography conditions (e.g., light conditions such as weather, and distance and angle to the targets) (Stevens et al. 2007; Sanz et al. 2013). Therefore, as a first step, categorical analysis of multiple spots/bands/patches, as used in this study, is preferable.

Although a web-based approach is cost-effective for describing large-scale patterns, it is important to recognize some limitations. First, inter-annual variation was not considered in this study, because I could not find enough photographs within years. To estimate the breeding period, photographs from before, during, and after the breeding period were needed: I could not estimate the breeding period in Chikuma River, mainly because of the lack of photographs in the spring. A balanced, relatively large dataset is needed to evaluate inter-annual variations (possibly 50–100 photographs per year based on the three rivers examined). If breeding period varies among years, and if the dace with nuptial coloration are more likely to be photographed than non-nuptial-colored dace, my analyses could overestimate the length of the breeding period. Second, non-random sampling is suggested, because the number of photographs was affected by human populations and relative abundance of *Ugui*. In addition, if rivers are long and the fish spawn in several places with different timing within rivers, estimation will be more difficult, because detailed localities are barely available from web images (only the names of rivers or towns, villages, and cities were available for most photographs). Another potential sampling bias is that uncommon phenotypes might be more likely to be photographed. This could be problematic if researchers are trying to evaluate the frequency of morphs or variations. However, it may be beneficial for describing overall variation if citizens tend to take photographs of rare phenotypes. Third, I subjectively categorized colorations, because qualitative evaluation of color hue from compressed photographs (e.g., jpeg and png) can have some biases (Stevens et al. 2007). Nonetheless, Leighton et al. (2016) subjectively evaluated color patterns of various animals using citizen photograph data on the web, and yielded results consistent with previous studies. I believe that evaluated patterns in this study were biologically meaningful, because the consistency with a previous study was high: over 80% of individuals were categorized with the same coloration as reported by Nakamura (1969). In addition, repeatability between persons was fairly high: consistency with another person was (i) 96% for judging the presence or absence of nuptial color, and (ii) 80.5% in categorizing color patterns. Higher repeatability in judging presence or absence than scoring coloration could be due to evaluation simplicity.

Some limitations will be rapidly overcome by methodological advances. In particular, by asking photographers to include a color reference and scale bars in high-resolution photographs (Casanovas et al. 2014), citizen-based science might allow me to quantitatively analyze color hue and investigate the effect of body size on coloration. Therefore, I started gathering photographs for scientific use on the web site "*Ugui* nuptial coloration project" (http://*Ugui*-guigui.wix.com/*Ugui*-guigui), in which I ask citizens to take pictures with some forms of scale information, such as tobacco cases and postcards. Finally, the reproductive behavior of *Ugui* can also be studied based on data from the web: over 40 videos on the spawning of *Ugui* and closely related species (*Maruta* and *Ezo*-*ugui*) were uploaded to YouTube as of 31 December 2016, which will facilitate comparative studies.

Potential factors that affect nuptial coloration

Although my nuptial coloration analyses were preliminary, several important findings were yielded: (i) color co-varied within black bands and red bands/patches but not between black and red bands/patches, (ii) nearly 20% of individuals lacked a few red bands/patches with some exceptional phenotypes (see Fig. 2f–h), and (iii) large color variations existed within rivers. I also found that variation in color pattern was greater in allopatric than sympatric areas with a closely related species, *Maruta*, which can potentially hybridize with *P. hakonensis* (Fig. 2j). Potential factors that affect nuptial coloration include the conditions of individuals, such as nutritional status (Craig et al. 2001); local environment, such as predation risk (Andersson 1994) and light conditions (Reimchen 1989), and timing (Kodric-Brown 1998).

Nuptial coloration of *Ugui* varied at a small spatial scale (i.e., within rivers). Red/orange coloration in *Ugui* is based on carotenoids (Matsuno and Katsuyama 1976), which animals can only acquire from diet (Matsuno 2001). Carotenoids are plant-synthesized pigments; thus, their abundance and coloration should co-vary with primary productivity (Leavitt 1993). *Ugui* migrate to the ocean or reside in rivers (Sakai and Imai 2005), which can shape substantial variation in body size within rivers (Nakamura 1969). Therefore, such tactics might result in a nuptial color variation via nutritional status, as is known in salmonids (Craig et al. 2001). In addition, because variations within spawning schools were small (I only found one or two phenotypes within each photograph), such variations may also be due to temporal change. For example, Nakamura (1969) stated that Rb3 occasionally existed in the non-breeding season, which indicates that each coloration component appears/disappears at different timing.

Together with variations within rivers, geographic variations in nuptial coloration may also exist, because unique phenotypes were found in some areas (Fig. 2f–h: central Honshu, northern Kyushu, and Tama River, respectively). Hybridization with *Maruta* might also change nuptial coloration, because the two species differ in the coloration: *Maruta* has one dark grey/black band on the lateral line and one red (not orange) band below the lateral line (Nakamura 1969; Sviridov et al. 2002; Sakai and Amano 2014). I found three samples with an intermediate phenotype in nuptial coloration: two obvious orange bands above and below the lateral line, and one dark grey band on the lateral line. These samples were photographed in Tama River (Fig. 2h), where both species are abundant. The number of pre-dorsal scales (33–35) was also intermediate between the two species: it was within the range of *Ugui* (29–36: Amano and Sakai 2014), but also the range of *Maruta* (34–41: the range of the subspecies *P. b. maruta*, Sakai and Amano 2014). Because cranial morphology of the three individuals resembled *Maruta* (cheek wider than eye diameter), these individuals may be *Maruta*. However, the presence of an orange band above the lateral line, which is a characteristic of *Ugui*, has never been reported in *Maruta* (Nakamura 1969; Sviridov et al. 2002; Sakai and Amano 2014). These individuals are potentially hybrids, although hybridization between the daces is rare in northeastern Honshu (Tohoku district: Hanzawa et al. 1984; Sakai et al. 2007). Further study utilizing genetic markers in unexplored regions, including Tama River, is required. If coloration in daces prevents heterospecific matings, as previously suggested (Gritsenko 1974), species-specific coloration should be more conserved in sympatric than allopatric areas, because there is no risk of hybridization in allopatric regions (i.e., reinforcement: e.g., Higgie and Blows 2008).

I also suggest that the nuptial coloration of *Ugui* may differ between Japan and Primorye, Russia: in Japan, red/orange spots were absent from black/grey bands and Rb2 rarely reached the caudal fin; alternatively, in Primorye, Sviridov et al. (2003) reported that spots tended to be present (approximately 40% of individuals surveyed) and Rb2 reached the caudal fin (approximately 90% of individuals). This could be due to either the conditional or environmental factors discussed above, or genetic differences; *Ugui* in the eastern Sea of Japan, including Primorye, are genetically different from those in the Japanese archipelago (Sakai et al. 2004; Polyakova et al. 2015).

Mutual ornamentation is taxonomically widespread, but its function is still debated (Kraaijeveld et al. 2007). Promising approaches include comparative studies that analyze inter- or intra-specific variations in mutual ornamentation or degree of sexual difference, and other ecological or social factors that affect natural/sexual/social selection. My findings regarding nuptial color variation within/between populations provides a foundation for investigating ecological/social factors that affect mutual ornamentation. In addition, some uploaders in western Japan suggested that nuptial coloration is more elaborate in males than females. Citizen-based science could facilitate studies that focus on intra-specific variation in strength of sexual difference, which is important for revealing the function of mutual ornamentation.

Appendices

Google Trends analysis

As the Web search frequency by citizens is suggested to be useful to estimate phenology and range of organisms (Ginsberg et al. 2009; Proulx et al. 2014), I investigated the breeding season and local abundance of *Ugui* based on the search frequency of "*Ugui*" obtained from Google Trends (hereafter GT, https://www.google.co.jp/trends/. Accessed 27 December 2016). Spatial data of GT from 29 November 2011 to 2016, and temporal data of GT from 27 December 2011 to 2016 were obtained.

Although GT value was 0 in 26 prefectures (22/26, Fig. S1A) where the dace existed in most of the areas according to the national census, GT value significantly correlated with abundance (r = 0.34, P < 0.05, Fig. S1B). When all data were combined, a seasonal trend appeared with the peak being early summer to early autumn (Fig. S2). This trend, however, disappeared when analyzing each prefecture independently.

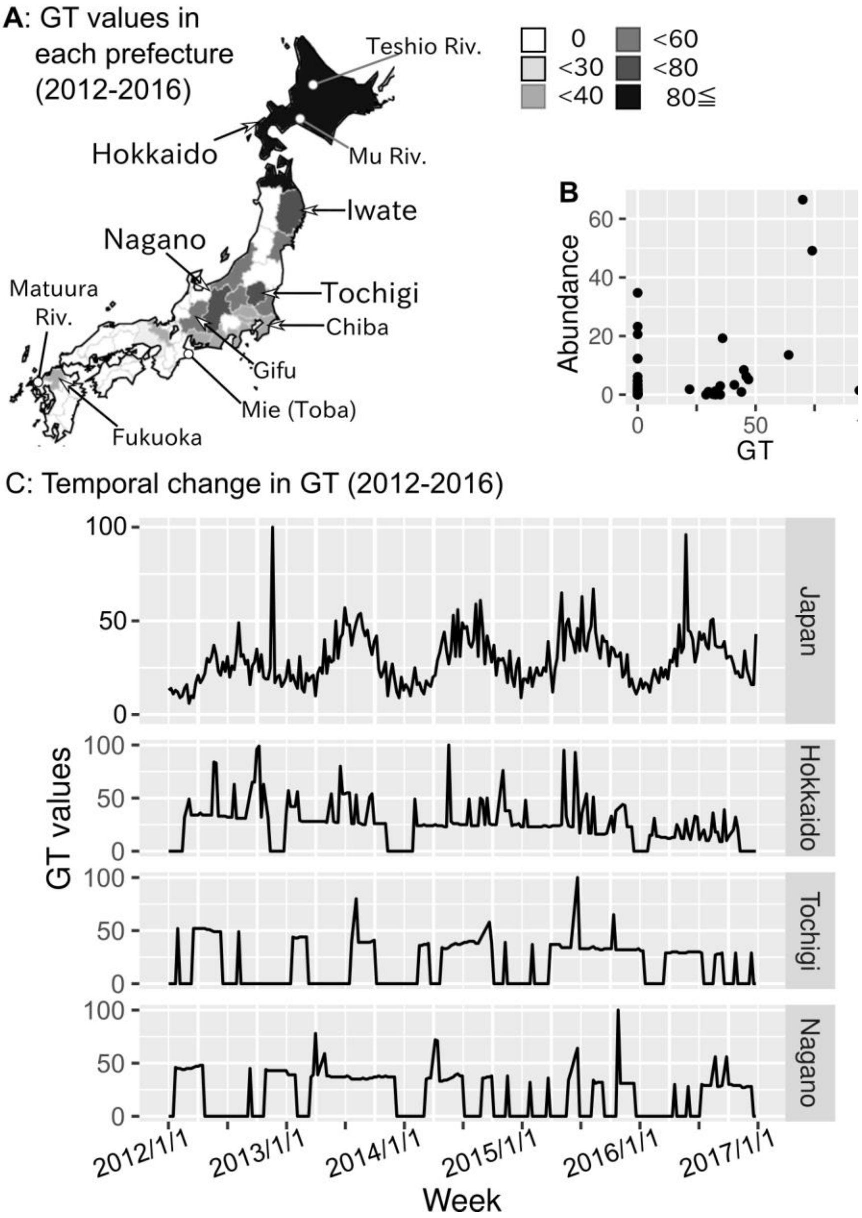

Figure S1 Spatial (**A**) and temporal (**C**) distributions of Web search frequencies (GT) of "*Ugui*", and the relationship between GT and relative abundance of *Ugui* based on national census (**B**). In **C**, temporal changes of GT values in Japan, Hokkaido, the highest search frequency with breeding period from May to late-July (Okada 1935; Ito 1975; Sakai 1995), Tochigi, the second highest frequency with the period from April to late-May (Iwamoto and Kanoki 1983), and Nagano, the fourth highest frequency with the period from mid-April to mid-July (Koyama and Nakamura 1955). Iwate prefecture, the third highest search frequency, is not shown because the breeding period have not been reported.

Chapter 3

Male species recognition based on female nuptial coloration

Because sexual selection typically acts on males, evolution of conspicuous ornamentation in females has been understudied. Genetic correlation between sexes and sexual or social selection on females have been proposed to explain female ornamentation, but they cannot fully explain observed patterns in nature such as female ornamentation in non-territorial, promiscuous species. The species recognition hypothesis, which postulates that ornamentation is adaptive because it prevents maladaptive hybridization, might plausibly explain female ornamentation. I examined the possibility of this hypothesis in two sympatric, non-territorial, promiscuous fish species. Both sexes of *Ugui* and *Ezo-Ugui* display species-specific conspicuous coloration in the breeding season. I conducted visual-based mate choice experiments using *Ugui* males and compared their association times between conspecific and heterospecific females. Males spent more time near conspecifics, indicating that *Ugui* males used visual cues to recognize conspecifics. Because the females presented to the males did not differ in body size, shape and behavior between the two species, the male preference for conspecifics was probably based on the females' nuptial coloration. These results suggest that female ornamentation may evolve or be maintained by not only sexual or social selection within a species but also interspecies interactions (e.g. hybridization).

Atsumi, K., Kishida, O., & Koizumi, I. (2019). Visual preference of males for conspecific mates in mutually ornamented fish: possible support for the species recognition hypothesis. *Journal of Ethology*, *37*(3), 353-362.

Introduction

Elaborate ornamentation has been well documented in male animals to have evolved through mate choice or intraspecific competition (i.e. sexual selection Darwin 1871; Andersson 1994). In many species across taxa, females also display elaborate ornamentation (Kraaijeveld et al. 2007). The emphasis on sexual selection in males has led to the notion that female ornamentation is non-adaptive; rather, it is merely a by-product of male ornamentation due to genetic correlations between the sexes (Lande 1980). This genetic correlation hypothesis requires male ornamentation, possibly evolved through sexual selection. However, a comparative study across >3,000 birds showed that the elaboration of female coloration positively correlates with male coloration, but not with male sexual selection which was quantified by social polygyny, male-biased sexual size dimorphism and lack of paternal care. Rather, male sexual selection leads to sexual dichromatism with bright colored males and dull colored females (Dale et al. 2015). Hence, male sexual selection alone may not explain the wide prevalence of female ornamentation. Alternatively, recent research on female ornamentation has stressed the importance of sexual selection in females (Kraaijeveld et al. 2007; Clutton-Brock 2007) as well as of badges of status, irrespective of sex, in reducing antagonistic social interactions (i.e. social selection Tobias et al. 2012). Both sexual selection and social selection, which are associated with intraspecific interactions, may be valid explanations for female ornamentation, but they cannot fully explain some patterns observed in the wild. For example, the comparative study above also showed that the elaboration of female coloration is weakly associated with female sexual and social selection (cooperative breeding was used as an indicator of social selection: Dale et al. 2015). In fishes, both males and females of non-territorial, promiscuous species often exhibit ornamentation (Lachner 1952; Sakai 1995; Sabaj et al. 2000), even though sexual selection and social interactions are expected to be weak in such non-territorial, promiscuous species. Thus, researchers should pay more attention to possible factors other than intraspecific interaction as an alternative driver of evolution of elaborate female ornamentation (or mutual ornamentation: both males and females have ornamentation).

More than 100 years ago, Wallace (Wallace 1889) proposed that conspicuous species-specific ornaments reduce harmful interspecific interactions such as hybridization and aggression by ensuring correct species recognition. This hypothesis has logical appeal for female/mutual ornamentation because selection against hybridization can act on sexual signals not only in males but also in females (Takakura et al. 2015). In other words, selection against hybridization can favor male mate species recognition based on female ornamentation. Yet, male species recognition has rarely been investigated in female or mutually ornamented species (Wiernasz 1995; Seehausen et al. 1999; Zoppoth et al. 2013), and previous studies do not experimentally excluded chemical or tactile communications. Hence, the species recognition hypothesis (Wallace 1889) has rarely been formally tested in the context of female ornamentation (but see Svensson et al. 2007; Pierotti et al. 2008).

Pseudaspius species provides an excellent opportunity to test species recognition hypothesis in female ornamentation. *Ugui* and *Ezo-Ugui* are morphologically similar, but both sexes exhibit species-specific conspicuous coloration only in their breeding season (i.e. mutual ornamentation). *Ugui* has three orange and two black horizontal bands on its head and along its flanks and orange fins, whereas *Ezo-Ugui* has red cheeks and fins (figure 1) (Nakamura 1969; Atsumi et al. 2018). Further, both species are non-territorial and they spawn in schools (Ito 1975; Sakai 1995). These behaviors may preclude the current major hypotheses proposed to explain female ornamentation (i.e. social selection, sexual selection in females, and the genetic correlation between sexes combined with sexual selection in males). Instead, the breeding ecology of *Ugui* and *Ezo-Ugui* suggests that the species recognition hypothesis is more likely to explain female ornamentation in these species. Although the breeding season and habitat of these two species overlap

(Gritsenko 1982; Sakai 1995), hybrid swarms have rarely been observed in the wild (Hanzawa et al. 1984; Sakai and Hamada 1985; Sakai et al. 2007). These findings suggest that these species discriminate between conspecifics and heterospecifics and thereby avoid hybridization. The aim of this study was to investigate the role of female nuptial coloration in sexually isolating these species. I examined whether (i) visible female traits enhanced *Ugui* male visual-based species recognition in a two-choice experiment, and (ii) visible female traits other than coloration (i.e. behavior and body size and shape) differ between the species. If *Ugui* males showed a preference for conspecifics based on visual cues and females did not differ behaviorally or morphologically between species, then female ornamentation (nuptial coloration) should be a key trait by which *Ugui* males discriminate between conspecific and heterospecific females.

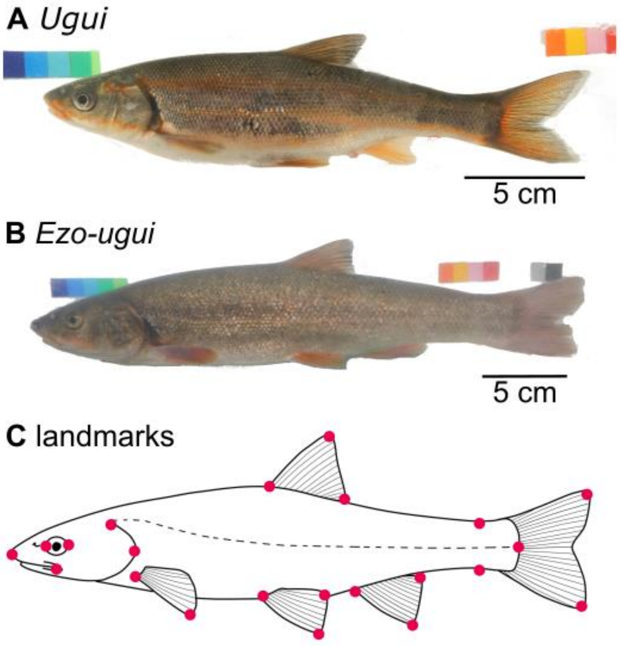

Figure 1 Nuptial coloration in females of **A** *Ugui* and **B** *Ezo-ugui* with color bars. Arrows indicate the positions of red/orange bands. **C** Positions of the 22 landmarks used in the geometric morphometrics analysis

Methods

Sample collection and rearing conditions

Fish used in the mate choice experiment were collected from Kotoni-Hassamu River in central Hokkaido, northern Japan (see electronic supplementary material, figure S1). I collected daces by using throw nets during their breeding season, from 18 May to 23 June 2017.

The collected fish were separated according to sex and species and kept in segmented reaches of an artificial channel next to the Horonai River in the Tomakomai experimental forest of Hokkaido University (see electronic supplementary material, figure S1) at 13–16 ˚C, the temperature range at which they spawn (Gritsenko 1982), under natural lighting conditions. Sex was determined by squeezing the abdomen, forcing the release of gametes from the gonoduct, and species were identified by their nuptial coloration. Individuals were marked for identification by clipping their fins in unique combinations. For one to seven weeks before the experiment, all collected fish were fed on pellet food once a day. All fish kept breeding coloration during the study period.

Mate choice experiments

I conducted the male mate choice experiment using *Ugui* males, not *Ezo*-*Ugui* males because of the small sample size of *Ezo-Ugui* males. An acrylic aquarium (250 cm × 100 cm × 100 cm deep, filled with fresh water to 30 cm depth) located in an outdoor facility was used as the experimental arena for the male mate choice experiment (figure 2). At each end of the arena, I placed a small glass aquarium (60 cm × 30 cm × 36 cm deep, water depth 30 cm). The bottom surfaces of both the arena and the aquaria were covered by gravel. A two-choice experiment was conducted in which one *Ugui* male was placed in the arena (outside the small aquaria), a *Ugui* female was placed in one of the two small aquaria (i.e., stimulus fish compartment), and a *Ezo-Ugui* female was placed in the other. Con- and heterospecific females were alternately presented in the left and right aquarium. This experimental setting allowed the male fish to use visual and acoustic information, but not chemical cues, to recognize the species of the females. I note, as sound-producing organs (e.g. drumming muscle surrounding swim bladder and spines on the fins: Amorim 2006) are not found in *Pseudaspius* species (KA personal observation), acoustic communication is less likely to occur. To evaluate male choice, I defined three resident zones: one adjacent to each stimulus fish compartment (‘‘association zones’’, within 50 cm of each end of the arena) and a middle ‘‘neutral’’ zone (more than 50 cm from either end) (figure 2). The focal male and stimulus females were put into the aquaria and allowed to acclimate for 2 min before the trial was started. As male behavior did not differ qualitatively between the early and late phase of the experiment (KA personal observations), I consider that acclimation time was enough. During each trial, I made video recordings of the behaviors of the male and females in the two association zones. Using the video recordings, I measured the male association time with each stimulus female for a total of 18 min. It was not possible to record data blind because my study involved focal animals. In 7 of 43 trials, the subject male did not visit either female during the 18-min experimental period. I considered that those individuals were not sexually active, or that they did not recognize either stimulus as female, and excluded those trials from the analysis.

Body size of subject males and stimulus females was measured as fork length (FL). Trials of 37 males (215.7 ± 34.3 mm, FL ± SD) were included in the analysis. Body size (FL) of the stimulus females (five *Ugui* and four *Ts*) used in these trials was not different between the species (*Ugui,* 192.2 ± 11.8 mm; *Ezo-Ugui,* 183.4 ± 7.3 mm; $P > 0.2$, *t*-test). In each mate choice trial, the difference in body sizes between the stimulus females was within 7%. The size difference between female pairs, as well as male body size, were considered in the statistical analysis.

Trials were conducted from 12 July to 17 July 2017 during daytime hours (i.e. 10:00–18:10). Water temperature was 16 ± 1 ˚C and light conditions were controlled by a roof over the outdoor facility (to reduce sunlight) and a 100 W fluorescent lamp. After each day's trials, the experimental arena and stimulus compartments were emptied, dried, and refilled with well water to reduce the potential effects of odors and chemicals from previous trials. Stimulus females were used multiple times and the possible effect of stimulus female pairs was accommodated for the analysis.

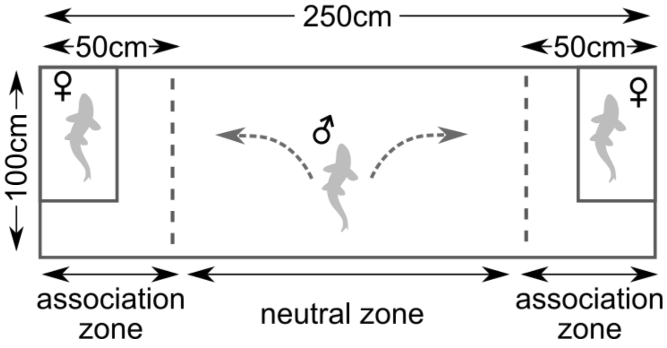

Figure 2 Diagram of the mate choice experimental set-up

Stimulus female behavior

Neither of these Far Eastern dace species show characteristic mating behaviors such as dancing (Ito 1975; Gritsenko 1982), nor did I observe such behaviors during my experiment (KA personal observation). Stimulus female behavior was evaluated from the video recordings by measuring horizontal position (0, far side from subject male; 1, middle; 2, near side), vertical position (0, bottom; 1, middle; 2, water surface), body angle with respect to the subject male (0, away; 1, vertical; 2, towards), and swimming activity (0, no swimming; 1, slowly swimming using fins only; 2, actively swimming by using fins and body undulation). Measurements were made at 1-min intervals during each 18-min mate choice trial. Thus, the four behavioral measurements were recorded 19 times in each trial.

Stimulus female morphology

The body shape of the stimulus females was analyzed by using a landmark-based geometric morphometrics analysis to quantify the overall shape of the body in lateral view. The shape was captured by 11 digitized landmarks generated using TpsDig 2.19 software (Rohlf 2010), selected to capture major body regions (figure 1C), from fish images obtained from the video clips. As the landmarks were made based on moving specimens, landmarks were set on cranial morphology and fin insertions that are relatively stable while fish swims.

Statistical analysis: male mate preference

As a measure of mate preference for conspecifics, I calculated the strength of the preference (SOP, e.g. (Tobler et al. 2008; Roberts and Mendelson 2017)) as follows:

$$\mathrm{SOP} = \frac{(\text{association time with conspecifics}) - (\text{association time with heterospecifics})}{(\text{association time with conspecifics}) + (\text{association time with heterospecifics})}$$

SOP is positive when the association time with conspecifics is longer, and is interpreted to indicate a preference for conspecifics.

To examine whether male *Pseudaspius* species prefer conspecific females on the basis of visual stimuli while controlling the effect of subject male body size and stimulus female pairs, a linear mixed model with normal errors (LMM) was fitted. The explanatory variables were (i) body size of subject males, (ii) body size difference between the stimulus females, represented as the ratio of conspecific body size to heterospecific body size, (iii) position of conspecific females (i.e. side effect) and (iv) their interaction. Stimulus female pair ID was included as a categorical random effect. All possible combinations of models using these predictor variables were compared using the BIC. In the best model, the statistical significance of each parameter was computed via the Satterthwaite approximation**.** I also evaluated the importance of those parameters by model averaging. All analyses were performed in R 3.4.3 (R Development Core Team 2019). I used following R packages: the lme4 package to construct LMMs; the MuMIn package to select LMMs and model averaging; and the lmerTest package to evaluate the significance of parameters within LMM,

Statistical analysis: female behavior

I conducted a PCA based on a correlation matrix to investigate variations in female behavior in the pooled data of the two species. The first two PCs successfully explained nearly 80% of total variance (PC1, 47.5%; PC2, 30.6%: table S1). I used the PC1 and PC2 values in the subsequent analysis.

To evaluate interspecies differences in behavior, I fitted an LMM to each behavioral PC value. The possible effect of female individuals and trials were accommodated as categorical random effects. The significance of each parameter was computed via the Satterthwaite approximation (Satterthwaite 1946)**.** I used the lme4 and the lmerTest packages of R.

Statistical analysis: female morphology

Landmark coordinates were aligned by a generalized Procrustes analysis, which centers, scales, and rotates landmark configurations to minimize the least-squares distance between shapes (Zelditch et al. 2012). The interspecies difference in body shape was evaluated by Procrustes ANOVA which assesses shape variation and covariation for a set of landmark coordinates aligned by Procrustes analysis (Klingenberg and McIntyre 1998). The geometric morphometrics analyses were conducted with MorphoJ 1.06d software (Klingenberg 2011).

Results

The total association time varied across trials (62.8 ± 31.1 % of total trial time). Males of *Ugui* preferred conspecific females on the basis of the visual stimulus: in the best model, SOP was significantly positive (0.41, $P = 0.014$, figure 3). The best model ranked by the BIC did not include the male body size, the female size difference, or their interaction (delta BIC > 3.8; table S2). The averaged model also showed no significant effect of the body size and gave similar estimates of SOP (0.40, $P < 0.05$; appendix S1 and table S3 in electronic supplementary material). On average, regardless of stimuli female pairs, association time with conspecifics was 11.7 ± 23.0 times (±SD), and 254.8 ± 423.7 s, longer during all 18-min mate choice trials.

I found no interspecies differences in stimulus female behavior and morphology. The LMMs fitted to the PC1–2 axes of behavioral data showed no significant behavioral difference between species (PC1, $P > 0.1$; PC2, $P > 0.8$: table S4 in electronic supplementary material). In addition, Procrustes ANOVA indicated no interspecies difference in body shape (*F* = 1.29, *P* > 0.2).

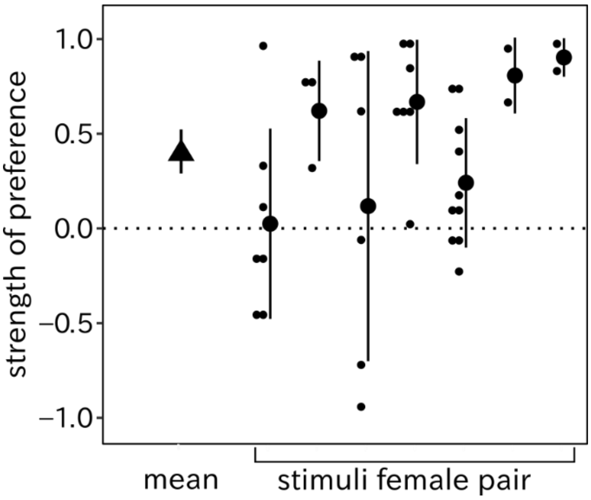

Figure 3 Visual-based male mate choice experimental results for each stimulus female pair (circles) and its overall mean estimated by the best linear mixed model determined by BIC model selection (triangle). For the results of each female pair, the black circles on the left side show strength of preference (SOP) of individual males. The bars indicate SD

Discussion

Although the species recognition hypothesis (Wallace 1889) can plausibly explain the mechanism driving evolution or maintenance of ornamentation in females as well as males, it has rarely been demonstrated previously (Svensson et al. 2007; Pierotti et al. 2008). The results of my experiment, conducted with two Far Eastern dace species in which the females display nuptial ornamentation, provide the support of the ability of this hypothesis to explain female ornamentation. The mate choice trials allowed the males to use visual information, but not chemical information, to recognize females. In these trials, *Ugui* males stayed near conspecific females 11.7 times longer with than they stayed near heterospecific females, indicating that male *Ugui* can discriminate two Far Eastern dace species visually. Potential visual signals or cues for male choice include body size, morphology, coloration, and behavior, or combinations of these. By using females of similar size as stimuli in the experiments, I precluded a possible body size effect on mate choice. Accordingly, LMM model selection and averaging inferred no effect of female body size. Furthermore, the results of my behavioral and morphometry analyses indicated that female behavior and morphology, at least as captured by my metrics, did not differ between the species. Thus, my experimental results suggest that female nuptial coloration is a signal that enables males to discriminate conspecific females from heterospecific ones. The species recognition hypothesis (Wallace 1889), which was originally proposed to explain the function of male ornamentation as a barrier against hybridization, may therefore also be valid for explaining female ornamentation.

In species with pair mating, it is well known that male mate choice based on female traits is important in maintaining species boundaries (Roelofs and Comeau 1969; von Schilcher and Dow 1977; Espinedo et al. 2010). In this study, I found that male mate preference exists even in species with promiscuous mating behavior. Because of such a mating system in *Pseudaspius* species, male mate choice may not function during actual spawning activity. Rather, male mate choice may play a role before spawning begins. For reproduction, individuals of these two species first aggregate into spawning schools. Thus, individuals may use the species-specific nuptial coloration to ensure their aggregation with conspecifics. Although the spawning habitats of these two species overlap, their spawning schools are largely segregated by species (Ito 1975). Visual cues may allow such fine-scale spatial segregation because they are fast and highly directional compared to chemical cues (Partan and Marler 2005; Gillam 2011). This spatial segregation means that individuals mate exclusively with conspecifics, even if their mate choices are random within the schools (Ito 1975). Thus, a visual preference for conspecifics might be a mechanism that prevents hybridization and allows these two Far Eastern dace species to coexist. Intraspecific spawning aggregations are taxonomically widely observed, especially in fishes (Domeier and Colin 1997; Anderson et al. 2016). It may be relatively common that mutual ornamentation ensures such aggregation and thereby reduces hybridization.

The evolution of female ornamentation has long been puzzling in evolutionary biology (Darwin 1871; Kraaijeveld et al. 2007; Tobias et al. 2012), and several explanations focusing on sexual and social selection have been proposed (Kraaijeveld et al. 2007; Tobias et al. 2012). Although the alternative hypotheses of the ornament function, i.e., sexual and social selection, were not experimentally excluded in this study, these processes may be weak in *Pseudaspius* species. Social selection should be precluded because *Pseudaspius* species are neither territorial nor aggressive throughout their life (Katano et al. 2010; Ogawa and Katano 2016). Generally, sexual selection can act in the dense species-specific spawning school through various processes such as alternative mating tactics (Suzuki et al. 2008), lek (Windle and Rose 2007) and scramble intrasexual competition (Byrne and Roberts 2004). However, sexual selection may be weak in *Pseudaspius* species because they are promiscuous and hardly aggressive (Ito 1975; Gritsenko 1982; Sakai 1995; Shirotori et al. 2006), which reduces opportunities for mate choice and intrasexual competition. Further, though the quantitative study is still awaited, the nuptial coloration seems not to follow the general patterns under sexual selection: the coloration does not vary with sexes and body size (Nakamura 1969; Sviridov et al. 2002, 2003). As only matured fish exhibit nuptial coloration (Nakamura 1969; Sakai 1995), nuptial coloration should show their maturity. Hence, species recognition, together with maturity recognition, may be an important function of the female nuptial coloration in these two Far Eastern dace species. Given that social interaction among *Pseudaspius* species is weak throughout their life-history (Katano et al. 2010; Ogawa and Katano 2016), the major benefit of species recognition is possibly to avoid hybridization, rather than to avoid harmful social competition. In general, distinguishing between conspecifics and heterospecifics benefits individuals, regardless of their sex, in a variety of taxa because it prevents harmful interspecific interactions (e.g., hybridization, interference competition, and predation) (Couzin and Krause 2003; Okamoto and Grether 2013). Therefore, species recognition where female ornamentation is used as a signal trait may be ubiquitous in nature.

My results may also partly explain the mechanism responsible for the observed geographic variation of nuptial coloration in *Ugui* across Japan. *Ugui* shows greater variation in nuptial coloration patterns in regions where it is allopatric with its hybridizing species (i.e. *Ezo-Ugui* and *Maruta*) than in regions where it is sympatric with those species (Atsumi and Koizumi 2017b). If the nuptial coloration of *Ugui* contributes to sexual isolation as suggested by the present study, selection against hybridization should favor species-specific color patterns in sympatric regions, thereby reducing the variation in nuptial coloration of *Ugui* in those regions. In allopatric regions, the absence of selection against hybridization might allow diversification of nuptial coloration. The geographic variation in coloration patterns might therefore reflect the absence or presence of interspecific interactions.

The species recognition hypothesis, and sexual and social selection hypotheses are not mutually exclusive, because ornamentation can convey multiple signals, including about species, quality as a mate, and social status, for species recognition, sexual selection, and social selection, respectively (Hill 2015). Further, these mechanisms may act interactively. For example, a small difference between species initially selected to prevent hybridization (i.e., species recognition) might later be enhanced by sexual selection (Mendelson and Shaw 2012). Or mutual ornamentation resulting from sexual or social selection might also function as an effective barrier to interspecific hybridization. Both theories and empirical studies have supported that disruptive or divergent sexual selection diverges male sexual trait and female preference thereby causing sympatric or allopatric speciation (Higashi et al. 1999; Seehausen et al. 1999; Maan and Seehausen 2011; Selz et al. 2016; Servedio 2016). Similarly, female ornamentation may diverge through disruptive/divergent sexual selection on females, or the combination of disruptive/divergent sexual selection on males and genetic correlation between sexes. Such interactive processes may drive the evolution of female ornament in *Pseudaspius* species because the selection against hybridization alone is probably not strong enough considering the high hatching rate of F1 hybrids (Atsumi et al. 2018). Considering the sexual or social selection and species recognition together, as well as genetic correlation, would further our understanding of the evolution and maintenance of female ornamentation.

Appendices

Appendix S1: Effect of the male and female body size on mate preference

The significant term in the averaged model was only intercept (table S2). The relative importance of each parameter, calculated through the model averaging procedure, was following: (i) male body size, 0.05; (ii) body size difference between females, 0.04; (iii) their interaction, <0.01.

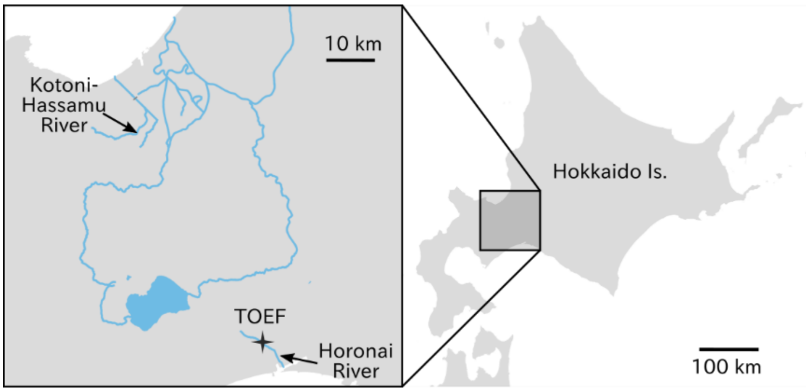

Figure S1: Sample localities. TOEF, Tomakomai Experimental Forest.

Table S1: Results of principal component analysis (PCA) on behavioural data

		PC1	PC2	PC3	PC4
Importance of PCs	Standard deviation	1.379	1.106	0.689	0.633
	Proportion of Variance	0.475	0.306	0.119	0.100
	Cumulative Proportion	0.475	0.781	0.900	1.000
Rotation of PCs	horizontal position	0.504	-0.499	0.433	-0.557
	vertical position	0.374	0.663	0.610	0.218
	activity	0.537	0.407	-0.637	-0.374
	angle to male	0.564	-0.382	-0.184	0.709

Table S2 Results of model selection on LMMs examining possible effect of male body size and female body size on preference for conspecific females

	explanatory variables									model selection			
	Intercept	**(A)** Subject male body size	[1]**(B)** Size difference between stimuli females	**(C)** Side	[2]A:B	[2]A:C	[2]B:C	[2]A:B:C	df	log Likelihood	BIC	delta	weight
Models ranked by Bayesian Information Criteria (BIC)	0.416								3	-28.585	68	0	0.795
	0.274			+					4	-28.685	71.8	3.81	0.118
	0.420		0.034						4	-29.724	73.9	5.89	0.042
	0.419	-0.020							4	-30.073	74.6	6.59	0.03
	0.262		0.041	+					5	-29.739	77.5	9.53	0.007
	0.277	-0.004		+					5	-30.151	78.4	10.36	0.004
	0.423	-0.028	0.040						5	-31.164	80.4	12.38	0.002
	0.243		0.249	+			+		6	-29.562	80.8	12.79	0.001
	0.310	-0.090		+		+			6	-30.537	82.7	14.74	0.001
	0.269	-0.011	0.043	+					6	-31.178	84	16.02	0

0.408	0.005	0.019		0.092				6	-32.117	85.9	17.9	0
0.256	-0.022	0.260	+			+		7	-30.969	87.2	19.21	0
0.301	-0.099	0.044	+		+			7	-31.537	88.4	20.35	0
0.201	0.037	0.018	+	0.115				7	-31.915	89.1	21.1	0
0.293	-0.120	0.287	+		+	+		8	-31.24	91.4	23.37	0
0.194	0.021	0.182	+	0.100		+		8	-31.855	92.6	24.6	0
0.233	-0.044	0.021	+	0.107	+			8	-32.352	93.6	25.59	0
0.232	-0.070	0.212	+	0.089	+	+		9	-32.214	96.9	28.93	0
0.174	-0.013	0.141	+	0.191	+	+	+	10	-32.536	101.2	33.18	0

Table S3: Results of model averaging on LMMs examining possible effect of male body size and female body size on preference for conspecific females.

	Estimate	*Adjusted SE*	*z value*	*Pr(>|z|)*
Intercept	0.416	0.133	3.135	0.0017
Body size of subject males	0.002	0.030	0.057	0.9544
Body size difference between stimuli females	-0.001	0.018	0.041	0.9673
Interaction	0.000	0.002	0.007	0.9943

Table S4: Estimated parameters in LMMs adopted to behavioural PC1-2 data.

	Parameters	Estimate	Std. Error	df	t value	Pr(>|t|)
PC1	Intercept	0.293	0.362	9.082	0.811	0.438
	Species (*Ts*)	-0.867	0.507	7.169	-1.711	0.130
PC2	Intercept	0.044	0.194	9.657	0.227	0.826
	Species (*Ts*)	-0.041	0.280	8.466	-0.146	0.887

Chapter 4

Shy males more strongly prefer conspecifics

Mating between different species is ubiquitous in nature. While environmental conditions and population density have been shown to affect the occurrence of heterospecific matings, much less is known about the potential role of individual characteristics. Here, I examined the association between boldness and mate choice to con- and heterospecifics in *Ezo-Ugui* which naturally hybridizes with congeneric species. I found a positive correlation between risk-taking tendency in an emergence test and a preference for heterospecific females. This finding suggests that bolder males may prefer novel phenotypes or are likely to make a miss-choice for their mating partners.

Atsumi, K., Koizumi, I. (in revision). Are bolder individuals more likely to choose heterospecific mates? A test in cyprinid fishes

Introduction

Animals often choose mates from other species, even when such mate choices result in unsuccessful reproduction (e.g. reduced offspring fitness and number). While heterospecific matings are often initiated by the environmental changes that hamper sexual communication (Seehausen et al. 1997; Fisher et al. 2006) and secondary contact of formerly geographically isolated species (Mallet 2005), heterospecific mating ultimately depends on individual mate choice (Rosenthal 2013). Mate preference shapes mate choice and thus is important in heterospecific matings – weak mate preference for conspecifics over heterospecifics will lead choosers to mate with heterospecifics. Mate preference for conspecifics may vary across individuals and correlate with major behavioral traits such as exploration and boldness (Sommer-Trembo et al. 2016). Studies investigating mate choice among conspecifics have revealed that mate preference consistently varies across individuals (Forstmeier and Birkhead 2004), and often correlates with boldness and exploration (Schuett et al. 2010; Rosenthal 2017).

Boldness – defined as the willingness to take risks (Réale et al. 2007) – may affect conspecific mate preference over heterospecifics through their positive reaction to novel stimuli or greater cognitive performance. Since boldness is associated with positive responses to novel objects (i.e. neophilia, $r = 0.4$, meta-analysis: Garamszegi et al. 2013), bolder individuals may respond more positively to unfamiliar courter signals (Rosenthal 2017). If heterospecifics are unfamiliar to choosers (e.g. due to habitat difference in the non-breeding season, or during secondary contact between species pairs diverged in allopatry), neophilia might result in a preference for heterospecifics. This hypothesis predicts a greater tendency for hybridization in bolder individuals. Conversely, bolder individuals may be less likely to hybridize considering their possible greater cognitive performance that can lead to more accurate mate choice. Although only a limited number of studies are included, a meta-analysis showed a positive association between accuracy in decision-making and boldness (Dougherty and Guillette 2018). Yet, despite the surge of interest in behavioral variation among individuals, few studies have investigated how boldness influences mate choice between con- and heterospecifics.

Here, I investigated the association between boldness and conspecific mate preference in cyprinid fishes, specifically *Pseudaspius* species (*Pseudaspius brandtii*, *Maruta*; *P. hakonensis*, *Ugui*; *Ezo-ugui*, *Ezo-ugui*). During the breeding season, *Pseudaspius* species form spawning schools largely segregated by species. However, pre-mating behavioral isolation among them is not complete: these three species hybridize mainly as a result of the sneaking behaviors of male *Ezo-ugui* in the spawning schools of the other species (Sakai 1995). It remains unclear why such irregular males exist. In order to understand the behavioral cause of hybridization in this fish system, I conducted an emergence test and mate choice trial between con- and heterospecifics (*Maruta* and *Ugui*) in *Ezo-ugui* males.

Methods

Sample collection and rearing conditions

I sampled for mature daces using cast nets and lure fishing during their breeding season in the Ishikari River system located in Central Hokkaido, northern Japan, in 2017. Stimuli females used in mate choice trials were collected in several rivers in Sapporo where the three species co-occur, from the 18th of June to the 17th of July (Appendix 1 and Fig. S1 for detailed localities). Subject *Ezo-ugui* males were collected in Lake Shikotsu, on July 29th. I collected *Ezo-ugui* males from an allopatric area where the other congeneric species do not inhabit. While sympatric populations should undergo reinforcement (selection against hybridization) that enhance mate preference for conspecifics and diminish inter-individual variation, allopatric populations do not undergo such reinforcement because there is no risk of hybridization. Hence, allopatric populations can harbor substantial variation in mate preference for conspecifics as compared to sympatric populations (Servedio and Noor 2003). An allopatric population is thus suitable for investigating the covariation of mate preference for conspecifics with behavioral traits. As in My previous study (Atsumi et al. 2019), the fish were separated according to sex and species, and kept in an artificial channel (each compartment 2.0 × 2.2 × 0.5 m, whole channel 12.0 × 2.2 × 0.5 m) next to the Horonai River in the Tomakomai experimental forest (Hokkaido University) at 13–16 ˚C. Body size of subject males and stimulus females was measured as fork length (FL). All collected fish were fed on pellet food once a day and maintained breeding coloration during the entire study period.

Emergence test

The emergence test was conducted once per fish for *Ezo-ugui* males ($N = 23$, Fork Length ± SD = 153.5 ± 17.1 mm) 2–4 days after they were caught. Each fish was gently placed into a covered box (refuge), set within an aquarium, with the hatch closed (see Fig. 1 for experimental apparatus). After 5 min of acclimation, the hatch was remotely opened, allowing the fish to emerge into the novel area compartment. The trials were recorded by a video camera (HC V360MS, Panasonic) set above the aquarium. From the video recordings, I recorded the elapsed time (=emergence time) for the subject to leave the refuge and enter the open environment for up to 60 min. A shorter latency of emergence from the refuge can be interpreted as greater boldness (Carter et al. 2013). Two individuals out of 12 that showed mate preference (see below) did not leave the refuge during this time. They were assigned a maximum score of 60 min. The aquarium was drained, dried and refilled before every trial. Both emergence test and mate choice experiments were conducted in an outdoor facility at the Tomakomai experimental forest during daytime hours (i.e. 10:00–18:10). At the facility, the water temperature was 16 ± 1 ˚C and light conditions were controlled by a roof over the facility and a 100 W fluorescent lamp.

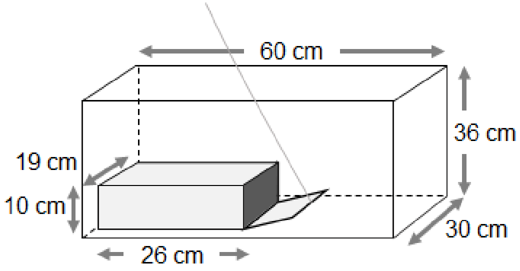

Fig. 1 Schematic representation of the emergence test set up. I used a glass aquarium (60 × 30 × 36 cm, water depth 10 cm) of which the bottom and side surfaces were covered by white sheets. A refuge, consisting of a wooden box (26 × 19 × 10 cm, grey shaded area) was set within the aquarium. The refuge had a hatch (19 × 10 cm) at its side, which was manually closable by means of a trap door manipulated by a thread (grey line).

Mate choice experiments

Both sexes of *Pseudaspius* species exhibit species-specific patterns in nuptial coloration which can enable mate recognition. Thus, I conducted a visual-based two-choice experiment following previously established methods (Atsumi et al. 2019). One to five days following the emergence test, two choice trials between con- and heterospecific females were conducted once per each *Ezo-ugui* male. For a mate choice arena, an acrylic aquarium (250 × 100 × 100 cm, water depth 30 cm) was used. Con- (*Ezo-ugui*) and heterospecific (*Maruta* or *Ugui*) females were alternately presented in the left and right glass aquarium (60 × 30 × 36 cm, water depth 30 cm). These aquaria placed at each end of the arena (Fig. 2 of chapter 3). The bottom surfaces of both the arena and the aquaria were covered by gravel. To evaluate male choice, I defined three resident zones: one adjacent to each stimulus fish compartment (‘‘association zones’’, within 50 cm of each end of the arena) and a middle ‘‘neutral’’ zone (more than 50 cm from either end). The focal male and stimulus females acclimated for 2 min before the trial was started. As male behavior did not differ qualitatively between the early and late phases of the experiment (KA personal observations), the acclimation time was deemed to be enough. I measured the male association time with each stimulus female for 18 min. Trials were conducted from the 1st to the 6th of August 2017. All aquaria were dried and refilled every day.

To avoid the possible effect of intraspecific variation among stimuli females on male choice, same stimuli female combinations were used for the two-choice trials (body size of females: in trials between *Ezo-ugui* and *Maruta*, 270 and 303 mm; in trials between *Ezo-ugui* and *Ugui*, 152 mm each). All females showed species-specific patterns of nuptial coloration during the trials.

Statistical analysis

As a measure of conspecific mate preference, I calculated SOP (Strength Of Preference) as the ratio of association time with conspecifics to total association time with both females. Greater SOP values indicate a stronger preference for conspecifics. To investigate the relationship between emergence time and SOP while accommodating for potential confounding effects, SOP was regressed by emergence time (minutes), heterospecific species used as stimuli females (i.e. *Ugui* or *Maruta*) and position of conspecific females (side effect: left or right), without any random effects. Since SOP is a continuous variable ranging from 0 to 1, I fitted a beta distribution to the regression. In 11 out of 23 trials, the subject male did not visit either one female, which was more frequent than My previous study using *Ugui* males (7 out of 43 males, Atsumi et al. 2019). *Ezo*-*ugui* males were less likely to visit both stimuli females presumably because they are less active (KA, personal observation) or shyer than *Ugui* males. I considered that those individuals did not recognize both stimulus females, and therefore excluded those trials from the regression. All statistical analyses were performed on R 3.6.1 (R Core Team and R Development Core Team 2019). The beta regression was conducted using package *betareg* (Grün et al. 2012).

Results

Males varied considerably in their emergence time from the refuge (all individuals tested, mean ± SD 39.74 ± 23.13 min, range 1.02 – 60 min, $N = 23$, nine individuals did not emerge; individuals used in the regression, 33.77 ± 24.54 min, range 1.02 – 60 min, $N = 12$, two individuals did not emerge). They varied greatly in SOP as well (all individuals tested, 0.614 ± 0.412, range 0.00 – 1.00, $N = 23$; individuals used in the regression, 0.594 ± 0.327, range 0.008 – 0.997, $N = 12$).

Beta regression showed SOP positively associated with male emergence time (slope ± SE, 0.043 ± 0.012, $z = 3.587$, $P < 0.001$, Fig. 2). SOP was higher when *Maruta* was shown as heterospecifics (1.609 ± 0.578, $z = 2.786$, $P < 0.005$, Fig. 2), presumably because *Ezo*-*ugui* vs. *Maruta* stimuli females pair had greater difference in body size (270 vs. 303 mm) compared to *Ezo*-*ugui* vs. *Ugui* stimuli pair (152 mm each). The side of the aquarium the conspecific female was placed did not significantly affect on SOP (-0.585 ± 0.779, $z = -0.750$, $P = 0.453$).

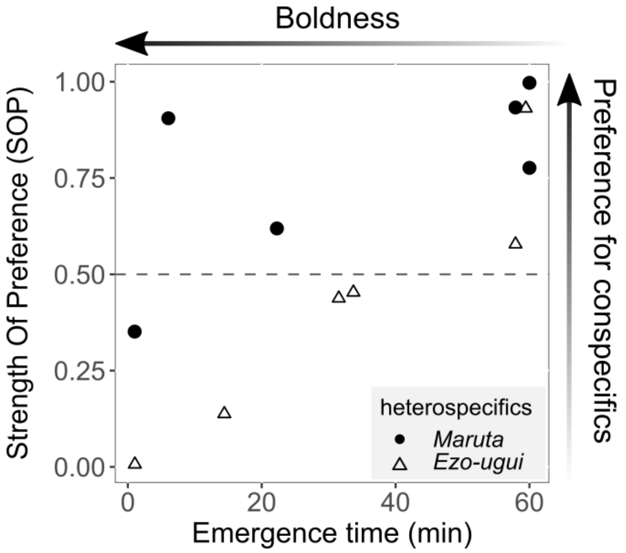

Fig. 2 Boldness negatively associated with the accuracy of species recognition. SOP is the ratio of the association time with conspecifics to the total association time with both females. *Maruta* (*Pseudaspius brandtii*) and Ugui (*P. hakonensis*) indicate heterospecific females used in mate choice trials.

Discussion

While the sample size was small, bolder males tended to show heterospecific mate preference whereas shy males showed a preference for conspecifics. To my knowledge, this pattern has never been described before. Cognitive basis underlying the association between mate preference and boldness remains unknown. At least, my results do not follow the prediction that bolder individuals are less likely to hybridize due to their higher cognitive performance (Dougherty and Guillette 2018). Rather, my result seems to follow the prediction that bolder individuals are likely to hybridize because they react more to novel stimuli (i.e. neophilia).

An alternative mechanism on the heterospecific mate choice by bolder individuals might be a simple mistake. Bolder individuals may show “fast” decision making with frequent errors (Sih and Del Giudice 2012), resulting in less accurate mate recognition. My results, however, may not match the prediction. If bolder males make a “faster” decision, boldness (shorter emergency time) will negatively correlate with the latency to approach females during the mate choice trial. In the beta regression accommodated heterospecific female species and aquarium side as fixed effects, emergence time did not affect latency to approach females (estimate ± SE of emergence time, 0.007 ± 0.012; $z = 0.59$; $P = 0.555$: Appendix 3). In addition, if bolder males made a faster decision than shy males, shy males might not have reached their decision within the timeframe in My experiment (i.e. 18 min). This was also rejected because the emergence time did not affect whether males visited both of the stimulus females or not, in the binomial regression accommodated heterospecific female species and aquarium side as fixed effects (estimate ± SE of emergence time, -0.034 ± 0.025; $z = -1.361$; $P = 0.174$: Appendix 4). Therefore, the weak conspecific preference of bolder males would not result from a simple “mistake”.

The link between boldness and mate preference for con- and hetero-specific have been overlooked, although boldness has been repeatedly shown to affect reproductive success (Smith and Blumstein 2008; Schuett et al. 2010) or cause assortative mating (Schuett et al. 2010; Ingley and Johnson 2014; Picq et al. 2019). More broadly, I know only little about how major behavioral traits such as boldness, exploration, aggressiveness and sociability (Réale et al. 2007), are associated with mate preference. To my knowledge, only two studies have tested the associations between mate preference for conspecifics and behavioral traits. Mate preference for conspecifics was positively correlated with exploration in female Atlantic mollies (Sommer-Trembo et al. 2016) but not with any behavioral traits in male sailfin mollies (activity, boldness, exploration, and sociability: Muraco et al. 2014). Even in mate choice within conspecifics, the associations between mate preference and behavioral traits have rarely been documented. In male Atlantic mollies, neither activity nor boldness was associated with male preference for larger females (Bierbach et al. 2015). In zebra finch, while David and Cézilly (2011) showed more exploratory females had weak preference strength, Schuett et al. (2011) found exploratory females strongly preferred exploratory males. More work is certainly required to understand the underlying mechanisms on how behavioral traits affect mate preference.

Since the emergence test and mate choice trials were not replicated, my results may not represent inter-individual behavioral variation or trait correlation that are stable across time and context (i.e. personality or behavioral syndrome, Sih et al. 2004; Réale et al. 2007). Some meta-analyses suggested that the repeatability of boldness or mate preference is less pronounced than other behavioral traits such as aggression and habitat preference (Bell et al. 2009; Holtmann et al. 2017). Nonetheless, I detected a clear association between emergence time and conspecific mate preference, which is of particular attention. Future studies should investigate the repeatability of boldness and mate preference across time and context in sympatric and allopatric populations of *Ezo-ugui*, as well as in the other hybridizing *Pseudaspius* species (*Ugui* and *Maruta*).

The association between boldness and mate preference might help explain why maladaptive hybridization among *Pseudaspius* species persists (F1 hybrids found but no hybrid swarm observed: Sakai 1995). In general, boldness affects predation risk and resource use (Réale et al. 2007), and daces are heavily predated by birds and consume eggs of congeneric species during the reproductive season (Sakai 1995; Kameda et al. 2010). Therefore, boldness during the reproductive season may be an important ecological trait that can affect survival and growth. If bolder males are favored in ecological contexts, they may be able to afford making a miss-choice within the context mating. The link between boldness and hybridization could be important in secondary contact because bolder individuals can spatially aggregate within the range edge (Sih et al. 2012). To understand the dynamics of hybridization, the link between conspecific mate preference and behavioral traits deserves further attention.

Appendices

Appendix 1. Sampling locality

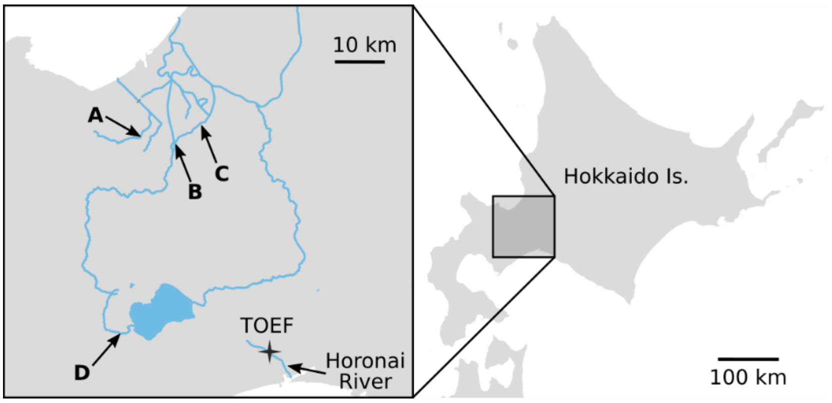

Figure S1: Sample localities (A–D) and study site (TOEF, Tomakomai Experimental Forest of Hokkaido University). A–C, sampling localities of stimuli females in Sapporo, sympatric area of the three species (A, Kotoni; B, Susukino; C, Kikusui); D, sampling locality of subject males, a tributary of Lake Shikotsu where only *Ezo-ugui* inhabits.

Table S1: Stimuli female pairs for the mate choice trials and their locality. For A–C of localities, see Figure S1.

	Species	Locality	Catch date	Body size (mmFL)

Ezo-ugui vs. *Maruta*	*Ezo-ugui*	B	19/07/2017	270
	Maruta	C	18/06/2017	303
Ezo-ugui vs. *Ugui*	*Ezo-ugui*	A	17/07/2017	152
	Ugui	A	25/06/2017	152

Appendix 3. Emergence time did not affect the latency to approach females

We conducted beta regression that the latency to approach females was regressed against the emergence time (minutes), heterospecific species used as stimuli females (i.e. *Ugui – Pseudaspius hakonensis* or *Maruta – Maruta*) and position of conspecific females (side effect: left or right). The model did not include any random effects. All of the 23 individuals used in the trials were included. The latency to approach females of which the individuals did not reach their decision ($N = 4$) was assigned as 18 min (maximum mate choice trial time).

We employed beta distribution because the latency to approach females has lower (0 min) and upper ranges (18 min). To apply beta distribution to the latency to approach females, I divided the latency by 18 min (upper limit), which makes the latency range 0–1. Here, beta-regression cannot be used to data including 0 and 1. I then transformed the latency by the following formula (Smithson and Verkuilen 2006):

$$\text{latency}' = \frac{latency(18-1)+0.5}{18}$$

We used the transformed latency to approach females above as a response variable in the beta regression. The beta regression was conducted on R 3.6.1 (R Core Team and R Development Core Team 2019) using package *betareg* (Grün et al. 2012).

The model showed, none of the emergence time, heterospecific female species and aquarium side significantly affected latency to approach females (Table S2).

Table S1. Beta regression showed emergence time did not affect the latency to approach females

Fixed effects	***Estimates ± SE***	***Z value***	***P***
Intercept	-1.081 ± 0.622	-1.739	0.082
Emergence time (min)	0.007 ± 0.012	0.59	0.555
Heterospecific females (Ugui)	-0.204 ± 0.619	-0.330	0.741
Aquarium side	0.827 ± 0.702	1.179	0.238

Appendix 4. Emergence time did not affect whether males visited both females

To see if shy males don't reach their decision within 18 min, I conducted binomial regression that whether the male visited both females was regressed against the emergence time (minutes), heterospecific species used as stimuli females (i.e. *Ugui – Pseudaspius hakonensis* or *Maruta – Maruta*) and position of conspecific females (side effect: left or right). The model did not include any random effects. All of the 23 individuals used in the trials were included. The regression was conducted on R 3.6.1 (R Core Team and R Development Core Team 2019).

The model showed, none of the emergence time, heterospecific female species and aquarium side significantly affected whether males visited both females (Table S1).

Table S2. Binomial regression showed emergence time did not affect whether males visited both females. I assigned 1 for the males visited both females, 0 for the males that did not visited either one female.

Fixed effects	***Estimates ± SE***	***Z value***	***P***
Intercept	2.472 ± 1.431	1.728	0.084
Emergence time (min)	-0.034 ± 0.025	-1.361	0.174
Heterospecific females (Ugui)	-1.007 ± 1.112	-0.906	0.365
Aquarium side	-1.213 ± 1.311	-0.925	0.355

Chapter 5

High hybrid viability in early-life-stage

Egg hatching rates of F1 hybrids among three fishes, *Ugui*, *Maruta*, and *Ezo-Ugui were* compared with pure crosses. They are deeply diverged (10–20 million years ago) but naturally hybridizing. Although crosses involving female *Ezo-Ugui* were not available, hatching success was high among all combinations of hybrids (76 ± 23 %) and comparable to pure crosses (86 ± 8 %), implying ecological or genetic isolation mechanisms maintaining each species in later life stages.

Atsumi, K., Nomoto, K., Machida, Y., Ichimura, M., & Koizumi, I. (2018). No reduction of hatching rates among F1 hybrids of naturally hybridizing three *Pseudaspius* species, genus *Tribolodon* (Cypriniformes, Cyprinidae). *Ichthyological research*, *65*(1), 165-167.

Introduction

In the process of speciation, it is generally assumed that fitness reduction of hybrid occurs first, often followed by premating (behavioral) isolation, and postmating (genetic) isolation will be strengthened (Coyne and Orr 2004). Divergence time required for the establishment of genetic incompatibilities in fishes varies from five to over 20 million years (myr) and generally 10 myr (Russell 2003). Despite the presumed old divergence (10–20 myr ago, Imoto et al. 2013), three species of *Pseudaspius* species (*Maruta*, *Ugui* and *Ezo*-*ugui*) can hybridize naturally, often with high frequencies (up to 20 %: Sakai and Hamada 1985). Hybrid swarms, however, have not been reported so far, indicating some isolation mechanisms. In these species, the mechanisms of reproductive isolation are largely unknown and strength of intrinsic isolation have not been investigated, whereas researchers have successfully reared *Maruta–Ugui* hybrids (Nakamura and Mochizuki 1953; Iwamoto and Kanoki 1983). Here, I investigated hatching rates of F1 hybrids among three *Pseudaspius* species, comparing with that of pure crosses.

Methods

I caught three males and two females of *Maruta*, three males and one female of *Ugui*, and three males of *Ezo*-*Ugui* in Abashiri River, eastern Hokkaido, Japan (43°82’ N; 144°09’ E), during their spawning season (6 July 2011). Three species sympatrically occurred and spawned below an artificial weir where daces were caught. At the time of sampling, breeding seasons of *Maruta* and *Ugui was* largely finished, whereas it was just before the beginning of the season for *Ezo*-*ugui*. Thus, females of *Ezo*-*Ugui was* unavailable. All of them were involved in both pure and two hybrid crosses, conducted at the Shibetsu Salmon

Museum where water temperature was 16.8 ˚C. Eggs were fertilized artificially, and hatching rate in each pair was investigated 12–15 days later because *Maruta* and *Ugui* is known to hatch five days after fertilization at 11–17 ˚C (Nakamura and Mochizuki 1953). Hatching rates were compared between pure and hybrid crosses using Welch's t-test.

Results

Hatching rates of F1 hybrids from all combination were generally high (>60–70 %) except for two crosses between male Ezo-*Ugui* and female Maruta (15–16 %) (Fig. 1). Hatching rates did not significantly differ between pure (0.86 ± 0.08) and hybrid crosses (0.76 ± 0.23) ($P > 0.05$).

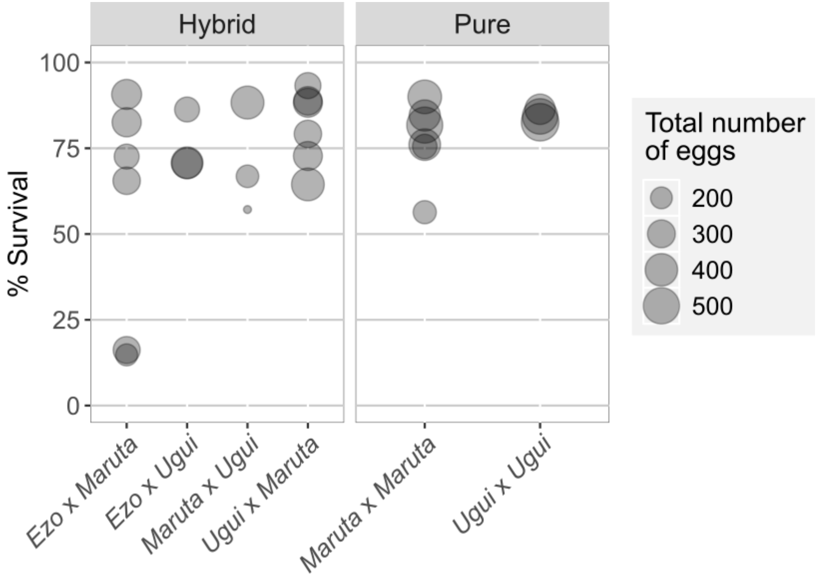

Fig. 1 Hatching rates of each F1 hybrid and pure cross among the three *Pseudaspius* species. Crossings are indicated as mother species × father species.

Discussion

Despite high hybrid fitness at least in early life-stage and large overlap in spawning season and habitat, hybrid swarms have rarely been observed in the wild (Hanzawa et al. 1984; Sakai and Hamada 1985; Sakai et al. 2007). These facts suggest that premating isolation is an important component of reproductive isolation among three *Pseudaspius* species. The lack of fertility reduction among three diverged daces (10–20 million years ago) is notable compared to a similar experiment in cichlids that fertilization and two-week survival rates of F1 hybrids decreased rapidly only after 1–4 myr divergence (Stelkens et al. 2010). As backcross hybrids of the daces exist in natural rivers (Sakai and Hamada 1985), F1 hybrids are not only viable but also fertile. Such low incompatibility might potentially lead genetic introgression among the three species.

The levels of introgression among the three daces remain unknown, but it seems that individual species are maintained despite hybridization (Sakai and Hamada 1985). Since hybrids are more or less viable in early life stages, extrinsic isolation should exist in later life stages. One of potential isolation mechanisms is the fitness reduction of hybrids during migration from freshwater to the ocean. The three species have different migratory tendency: all *Maruta* migrate to the ocean, all *Ezo-Ugui* are reportedly stream resident in Japan, and *Ugui* is partially migratory (some migrate to ocean and some reside) (Sakai 1995). Hybrids involving *Ezo-Ugui* might be maladaptive in terms of ocean migration. In addition, it is also known that fitness of later generation hybrids decreases dramatically due to deleterious interactions among parental alleles (i.e. hybrid breakdown: Stelkens et al. 2015), which may be also the case for the daces (Sakai 1995).

Chapter 6

General discussion

Premating or behavioral isolation is a widespread and powerful mechanism to maintain species diversity and reduce harmful hybridization (Rhymer and Simberloff 1996; Gröning and Hochkirch 2008). The roles of male mate choice on behavioral isolation have been overlooked because most (intra-species) sexual selection studies follow the "indiscriminate males and choosy females" rule. I demonstrated male preference for conspecifics by using mutually ornamented fishes that exhibit divergent breeding colorations among closely related species. I also showed the heterogeneity of male preference, another overlooked element, for con- and hetero-specific mating. Specifically, individual boldness, a plausible target for natural selection, affected mate choice. The findings of this thesis provide several new insights into the mechanisms of mutual ornament evolution.

Reproductive isolation mechanisms of *Pseudaspius* species in Hokkaido

In my thesis, I partially revealed reproductive isolation mechanisms among three congeneric species of *Pseudaspius* in Hokkaido. First, I suggest that the nuptial color pattern of *Ugui* was more strongly conserved in sympatry than in allopatry (Chapter 2). This implies that the nuptial coloration aid species recognition during the reproductive period. I then experimentally showed that male mate choice based on female nuptial coloration contributes to premating behavioral isolation between *Ugui* and *Ezo-Ugui*

(Chapter 3). Visual-based species recognition of *Ezo-Ugui* males highly varied among individuals and potentially correlated with their personality (Chapter 4). Other than the visual-based conspecific preference, chemical-based species recognition has been also suggested as premating isolation: males of *Ezo-Ugui*, at least, can discriminate species by using male urine and odor of egg (Sakai and Yoshii 1990). In addition, spawning season and microhabitat site selection, while these greatly overlap among the three species, may partially contribute to premating isolation (Ito 1975; Sakai 1995). Collectively, multiple mechanisms – including the visual-based conspecific preferences – should develop to prevent hybridization among the ecologically similar *Pseudaspius* species. On the other hand, I found no evidence of postmating isolation at least in the early life stages (Chapter 4). Similarly, other previous studies have failed to detect fitness reduction in hybrid offspring (Nakamura and Mochizuki 1953; Sakai 1995). Therefore, premating isolation may play a major role in the maintenance of distinct species.

My results, together with previous studies, are puzzling because postmating isolation seems lacking but premating isolation exists even though it has been believed that premating isolation evolves after postmating isolation (Coyne and Orr 2004). There could be two non-mutually exclusive hypotheses. First, premating isolation evolved independently from postmating isolation, through divergent/disruptive selection. Second, hybrids may perform worse in their late life-history stages such as growth, survival and reproduction: we have merely missed fitness reduction in nature.

Under the divergent/disruptive selection, populations can differentiate without internal postmating isolation (genetic incompatibility) (Nosil et al. 2009; Safran et al. 2013). Theoretically, either ecological or sexual selection can initiate speciation. When ecological traits correlate with sexual traits or mate preference, divergent/disruptive ecological selection will lead to divergence in sexual traits or mate preference, altimetry resulting in speciation (i.e. ecological speciation) (Nosil et al. 2009). Sexual selection can also facilitate speciation even without postmating isolation if female preference diverges within/across populations (Higashi et al. 1999; Servedio 2016). The striking ecological difference among *Pseudaspius* species is the difference in ocean migration tactics (see below). By altering food resources, the difference in ocean migration tactics potentially diverges nuptial coloration and chemical cue across *Pseudaspius* species because such characteristics are often sensitive to food resources (Fujii 1969; Grether et al. 1999; Ward et al. 2011; Otte et al. 2016). Therefore, both ecological divergence and divergent sexual selection across *Pseudaspius* species, if present, potentially lead to speciation in the absence of postmating isolation.

Although this and previous studies (e.g. Iwamoto and Kanoki 1983; Sakai and Hamada 1985) have not found direct evidence on postmating isolation in *Pseudaspius* species, hybrids may suffer fitness reduction. Since they are deeply diverged (10–20 Mya: Imoto et al. 2013), genetic incompatibility should accumulate among them. Iwamoto and Kanoki (1983) suggested that *Ugui* female × *Maruta* male hybrids are fertile, but the reciprocal hybrids are infertile. They found that both reciprocal hybrids from *Ugui* × *Maruta* grow faster than *Ugui*. However, *Maruta* female × *Ugui* male hybrids never exhibited nuptial coloration within the three years of their rearing experiment, whereas the reciprocal cross and *Ugui* exhibited the nuptial coloration at age 2 (Iwamoto and Kanoki 1983). Therefore, the reproductive success of F1 hybrids might be lower than parental species though the postmating isolation is not complete. Alternatively, migration to the ocean might reduce hybrid fitness. The three species vary in their ocean migration. While all individuals of *Maruta* migrate to the ocean at age 0+, *Ezo-Ugui* usually does not migrate to the ocean in Japan (Sakai 1995). The migratory tactic of *Ugui* is facultative (Sakai and Imai 2005; Ishizaki et al. 2010) and vary across geographic regions (Yamada & Koizumi, *unpublished*). Along with migratory tactics, three species vary in their salinity tolerance (Sakai 1995). If salinity tolerance and migratory pattern differently inherit (e.g. dominance and parent-of-origin effect), mismatch in salinity tolerance and migratory pattern should reduce hybrid fitness. While the migratory pattern may be under control of nuclear DNA, mitochondria genotype may influence salinity tolerance because mitochondria rich cells play a vital role in salinity acclimation (Kaneko et al. 1999; Sokolova 2018). Indeed, guppy shows maternal inheritance in salinity tolerance (Shikano and Fujio 1998). Nuclear loci are inherited from both parents, but mitochondria are usually inherited exclusively from mothers. Thus, hybrids from different migratory tactics and salinity tolerance – especially between *Maruta* male and *Ezo-Ugui* female – might suffer significant fitness reduction through the mechanism. More empirical researches comparing fitness of pure and hybrids species are certainly needed.

Intersexual correlation in elaborated traits: Favored condition and evolutionary implications

Evolution of mutual ornament – both males and females show conspicuous exaggerated phenotype – is a long mystery of evolutionary biology (Darwin 1871). In this thesis, I suggested that female ornamentation can reduce the maladaptive inter-specific hybridization by effectively showing the species of females. This species recognition hypothesis – ornamentation is beneficial by indicating species identity – has received rich empirical support on male conspicuous traits (Sætre et al. 1997; Seehausen and Van Alphen 1999; Boughman 2001; Collins and Luddem 2002). This thesis first linked the species recognition hypothesis (Wallace 1889) with the evolution of mutual ornamentation.

A major critic for the species recognition hypothesis is that ornamentation is not necessary for indicating species identity (Hill 2015). Although interspecies divergence in exaggerated traits should assist species discrimination especially in organisms that rely on visual information, species recognition hypothesis alone may not explain the evolutionary process of mutual ornamentation. Here I first review the potential evolutionary process of mutual ornamentation and raise a hypothesis that species recognition shapes mutual ornamentation together with the other potential processes. I then argue why *Pseudaspius* fishes developed species-specific mutual ornamentation. Finally, I argue the impact of mutual ornamentation on species diversification.

Candidate processes for the evolution of mutual ornamentation

In many mutually ornamented species, exaggerated phenotype is similar between sexes (Tobias et al. 2012) as in *Pseudaspius* species. This implies that intersexual genetic correlation explains "how" mutual ornamentation evolves (Lande 1980; Dale et al. 2015). Yet, genetic correlation alone does not explain evolutionary patterns of mutual ornamentation. Although intersexual genetic correlation is a common genetic architecture (Poissant et al. 2010), mutual ornamentation is not the norm – either one sex is exaggerated or both sexes are dull in many species (Kraaijeveld et al. 2007; Tobias et al. 2012). Explaining "why" mutual ornamentation evolves is necessary to understand its evolutionary patterns.

A classic hypothesis is that mutual ornamentation is just a by-product of male sexual selection (Darwin 1871; Lande 1980). Sexual selection on males can favor exaggerated traits in males, which develops the genes elaborating mating traits. Such genes can be expressed also in females because many genes are shared between sexes. In this scenario, ornamentation is not adaptive in females. Ornamentation, however, is often costly due to its energy demands and/or increased predation pressure (Hill 1999; Basolo and Alcaraz 2003; Godin and McDonough 2003; McGraw et al. 2005; Husak et al. 2006). Such natural selection against exaggerated traits should favor dull females, resulting in sexual dimorphism/dichromatism. Non-adaptive female ornament may be allowed to evolve if natural selection against ornamentation is weak. The cost of ornamentation can depend on species' trophic niche and architecture of ornamentation that influence predation risk. Comparative analysis across passerine birds found a positive association between body size and exaggerated female coloration, implying that reduced predation pressure in larger species allows female ornamentation (Dale et al. 2015). Seasonal color change also allows exaggerated female coloration: another comparative analysis across birds showed that seasonal color change is likely to evolve under high predation pressure (McQueen et al. 2019), which in turn indicates that temporal color change mitigates survival cost of elaborated coloration (e.g. nuptial coloration and biannual molt). Although a classic 'by-product' hypothesis is likely to require relaxed selection against ornamentation, studies linking mutual ornament evolution and survival cost of ornamentation are still rare and most of them exclusively focused on birds. Comparative studies examining this possible relationship in other taxon (e.g. fishes and insects) are awaited.

Another hypothesis is that selection can favor elaborated traits in both sexes (Kraaijeveld et al. 2007; Tobias et al. 2012). Most intuitively, mutual sexual selection – sexual selection simultaneously acts on males and females – could elaborate ornamentation if exaggerated phenotype is attractive to both females and males (Kraaijeveld et al. 2007). Mutual sexual selection is likely to operate in monogamous species where mutual mate choice occurs (i.e. both males and females are picky in mate choice) (Clutton-Brock 2007). Selection arising from any social interaction among individuals (e.g. aggression to obtain or defend social dominance or territory) – termed as social selection – can also exaggerate ornamentation irrespective of sex if ornamentation indicates individuals' ability in social competition (Tobias et al. 2012). Social selection is likely to act in species with complex societies. A review across birds stressed that social competition for territories after copulation (social selection), not mates (sexual selection), is an important evolutionary driver of female ornamentation (Tobias et al. 2012). Finally, natural selection can also favor elaborated ornaments irrespective of sex if exaggerated traits act as aposematic signals in both sexes (e.g. poison frogs and lionfishes) (Summers et al. 2015).

As newly suggested in this thesis, selection against hybridization can favor interspecies divergence in mutual ornamentation. This could be particularly important in species with mutual mate choice. In other cases – if intrasexual competition for mates (i.e. sexual selection) is heavily biased to one sex – the sex will be indiscriminate in choosing mates. This weak mate preference in one sex could relax selection favoring interspecies divergence on the other sex (Takakura et al. 2015). In such case, mutual ornamentation and its interspecies divergence may not easily evolve. Although my focus was the adaptive significance of ornamentation in reducing hybridization, species recognition could also be beneficial in any life-stages by reducing interspecies harmful social interaction or assisting conspecific aggregation. Since aggression is costly, interspecific aggression should be non-beneficial if the resource (e.g. food and mates) are partitioned between sympatric species. To reduce non-beneficial interspecies aggression, selection will favor interspecies phenotypic divergence so that individuals can discriminate species of potential competitors (i.e. antagonistic character displacement) (Okamoto and Grether 2013). In species that involve social competition in both sexes, selection can favor species recognition in both sexes. Conspecific aggregation will also confer several fitness benefits (i.e. Allee effect). Since potential mates will aggregate each other, individuals can increase fertilization success and reduce mate searching costs (Gascoigne and Lipcius 2004; Gascoigne et al. 2009). Further, by forming conspecific spawning aggregation, individuals can reduce hybridization risk even if they indiscriminately mate each other within the aggregation. Individuals may use the species-specific ornamentation to ensure their aggregation with conspecifics. Thus, interspecies divergence in mutual ornamentation can be favored in promiscuous species forming spawning aggregation because it can confer Allee effect and reduce hybridization. I note that aggregation can reduce predation risk but it does not require species recognition because such aggregation often consists of multiple species sharing predators (Martínez et al. 2018; Meise et al. 2020).

Importantly, sexual selection, social selection and selection favoring aposematic signals are not mutually exclusive, as in species recognition hypothesis. Although species recognition alone may be insufficient to lead to mutual ornament evolution, it can shape ornament evolution jointly with the other processes. For example, mutual ornamentation initially developed as a by-product of male sexual selection under the relaxed selection against ornamentation can be co-opted as species recognition traits. Alternatively, mutual ornamentation favored through mutual sexual selection or social selection can be co-opted as species recognition traits. This scenario is likely because species recognition can be important in species with mutual mate choice or social interactions. As shown in sexually monomorphic poison frogs (Yang et al. 2016), aposematic signals can be co-opted as species recognition traits.

Why Pseudaspius *species developed mutual ornamentation?*

As a potential evolutionary driver of mutual ornamentation, I exclusively focused on species recognition hypothesis in reducing hybridization and did not test other hypothetical processes. Still, social selection should be precluded because *Pseudaspius* species are neither territorial nor aggressive throughout their life (Katano et al. 2010; Ogawa and Katano 2016). Further, sexual selection may be weak in *Pseudaspius* species because they are promiscuous within large spawning aggregation and hardly aggressive during reproduction (Ito 1975; Gritsenko 1982; Sakai 1995; Shirotori et al. 2006), which reduces opportunities for mate choice and intrasexual competition. Rather, mutual ornamentation showing species identity can be beneficial in forming conspecific spawning aggregation. I did not measure the cost and benefit of ornamentation in survival. Still, the survival cost of nuptial coloration would be small because matured *Pseudaspius* fishes are large (mostly > 15cm FL) (Sakai 1995; Atsumi and Koizumi 2017a) and thus they have few predators in the aquatic community. A major natural enemy of *Pseudaspius* fishes would be birds (e.g. crows, herons and seagulls in Sapporo). It is intriguing that although spawning aggregation of *Pseudaspius* fishes dwells in shallow water, the number of predatory birds consuming *Pseudaspius* fishes are few (KA, personal observation). It seems that such birds do not actively feed them because of large body size or perhaps, bad taste. If this is the case, nuptial coloration may not be costly in survival. Rather, nuptial coloration would act as an aposematic signal. This hypothesis is awaited to be examined.

Mutual ornamentation may fuel diversification

In chapter 3, I found that *Ugui* males preferred conspecific females over *Ezo-Ugui* females visually, presumably based on female nuptial coloration. Although I did not investigate female species recognition based on male ornamentation, female choice is taxonomically widespread and reported elsewhere (Rundle and Schluter 1998; Veen et al. 2001; Pfennig 2007). Females of *Ugui* are thus likely to recognize species of males based on male nuptial coloration which is quite similar to that of females. If both sexes exhibit identical ornamentation, species recognition in both sexes may easily evolve because inter-sexually shared mate preference should more easily evolve than sexually different preferences. Here, mutual ornamentation can efficiently strengthen reproductive isolation. This scenario will be more likely when both sexes share alleles in mate preference loci and ornamentation loci (i.e. intersexual genetic correlation). Thus, inter-sexual genetic correlation can fuel the development of behavioral isolation and ultimately diversification.

This hypothesis would contribute to the understanding of the sexual selection – species diversity relationship (Barraclough et al. 1995; Gage et al. 2002; Tsuji and Fukami 2019). In theory, sexual selection is expected to facilitate diversification (Higashi et al. 1999; Safran et al. 2013). Comparative studies aiming to examine diversification through sexual selection have mainly used sexual dimorphism (or dichromatism) as a surrogate of sexual selection (Dale et al. 2015; Pigot et al. 2018; McQueen et al. 2019). Researchers have thus expected a positive relationship between sexual dimorphism and species diversity. Yet, they have yielded conflicting patterns so far – positive, negative and no significant association between them (Tsuji and Fukami 2019). Based on my hypothesis, intense diversification can occur even in sexually monomophic mutually ornamented species. This might have obscured the patterns in previous studies, which used the sexual dimorphism as a surrogate of sexual selection. The possible relationship between mutual ornamentation and diversification is awaited to be examined together with further examination of sexual selection and diversification relationship.

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