

ギンイソイワシ *Hypoatherina tsurugae* の温度依存型性決定機構に関する研究

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序章

魚類の性は多くの場合、受精時の「性染色体の組合せ」という先天的要因によって、遺伝的に決定されると考えられている。例えば、XX/XY 型の性決定様式を持つ種では、遺伝型が XX の個体は卵巣を保持する雌に、XY の個体は精巣を保持する雄に性が分化する。一方で、数種の魚類では、性決定および性分化が発生初期に経験した水温の影響を受けるという現象が確認されている。1981 年に北米原産のトウゴロウイワシ目魚類である大西洋産シルバーサイド *Menidia menidia* において、孵化直後の性決定時期に低温を経験した集団は雌に、高温を経験した集団はオスに性が偏るという「性の温度依存性」、すなわち温度依存型性決定 (Temperature-dependent sex determination ; TSD) の存在が魚類で初めて報告された (Conover and Kynard 1981)。現在では、メダカ *Oryzias latipes* やヒラメ *Paralichthys olivaceus* など他の分類群を含む約 60 種で「性の温度依存性」が確認されており (Baroiller, D’Cotta, and Saillant 2009; Kitano et al. 1999; Ospina-Álvarez and Piferrer 2008; Sato et al. 2005)、魚類の性はこれまで多くの研究者が考えてきた以上に、性分化時に経験する環境水温の影響を受けやすいのではないかと思われる。

20 世紀半ばから、世界規模で海水温上昇が観測されている (Intergovernmental Panel on Climate Change 2015)。とりわけ日本近海では、世界全体平均の海水温上昇率の約 2 倍となる 1.14°C/100 年の上昇が報告されている (Japan Meteorological Agency 2020)。また北太平洋海洋モデル NPOGCM をもとに算定された A1B シナリオに基づく 1981~2100 年の日本近海における年平均海面水温上昇率の予想は 2.0~3.1°C/100

年となっており、今後も日本近海の海面水温は漸進的に上昇すると考えられる (Japan Meteorological Agency 2008)。このような長期的な水温の上昇傾向のみならず、近年、短期間での急激な水温上昇も確認されている。例えば、2016年8月中旬の10日間に観測された日本近海の表面水温は、平年と比較して高い海域が多く確認された。特に日本海南部では平均海面水温 28.4°C、平年差+2.7°Cを示し、解析記録のある1982年以降では、年間を通して最も高い水温であったとされている。このような急激な水温上昇は、上空の気圧の変動とそれに伴った日照時間の増加、および海面付近の風量の減少に起因していると考えられている (Japan Meteorological Agency 2016)。さらに、20世紀半ば以降、日中の最高気温が平均最高気温を 5°C以上上回る日が5日間以上連続する「熱波」と呼ばれる現象の発生回数が、調査が行われた陸域のほとんどで増加していることが報告されている (Stocker et al. 2013)。このような気候変動は、海水温へも影響を及ぼすと考えられるため、今後も局所的・短期的かつ急激な海水温の変動が生じる可能性は十分に考えられる。

地球温暖化・気候変動に起因する漸進的または、急激な水温変動が生じると、前述の性が水温の影響を受ける魚種では、性決定時期の仔稚魚がその異常水温に晒されることで性が影響を受け、雌雄比の偏りが生じると考えられる。著しい性の偏りは、その生物集団の再生産能力の低下・資源量の減少を引き起こし、最終的には当該種の絶滅のみならず、周辺生態系へも悪影響を及ぼしかねない (Ospina-Álvarez and Piferrer 2008; Yamamoto et al. 2019)。さらに、近年これまで性が遺伝的に決定し水温の影響を受けにくいと考えられていた多くの魚種においても、性決定が水温の影響

響を受けることが示されているため、地球温暖化・気候変動に起因した異常水温による雌雄比の偏りが、将来的に日本沿岸に生息する多くの魚種で生じてしまう可能性が危惧される。一般的に、このような環境要因が野生の生物集団へ与える悪影響は不可逆的であり、一度生じるとその復元は非常に困難である。これを予防するためには、まずは魚種別に性の温度感受性を調査することが重要であるが、現在、「性の温度感受性」が調べられている魚種は約 60 種に過ぎず (Ospina-Álvarez and Piferrer 2008)、野生環境下はおろか飼育環境下においても殆どの魚種で明らかになっていないのが現状である。

本研究では、性決定が水温の影響を特に受けやすいと考えられる種として、トウゴロウイワシ目魚類に着目した。トウゴロウイワシ目魚類は先にも述べた通り、魚類で初めて「性の温度感受性」が報告された分類群であり、淡水域から海水域まで世界中に広く分布している。現在までのところ、新大陸に生息する *Odontesthes* 属 (Strüssmann, Calsina Cota, et al. 1996; Strüssmann, Hanke, et al. 1996)、*Chirostoma* 属 (Corona-Herrera et al. 2016)、*Leuresthes* 属 (Brown, Baumann, and Conover 2014)、*Menidia* 属 (Conover and Kynard 1981; Middaugh and Hemmer 1987) の 4 属で温度依存型性決定機構を保持する種が確認されており、本目魚類の「性の温度感受性」は他魚種と比較して高いと考えられる。

一方、日本沿岸海域にはギンイソイワシ *Hypoatherina tsurugae*、トウゴロウイワシ *Atherina valenciennesi* 等 8 種のトウゴロウイワシ目魚類が生息しているが (Sasaki et al., 2014)、その「性の温度感受性」の強度は全く明らかになっていない。

しかし近年、日本に生息するギンイソイワシにおいて雄性決定遺伝子の候補である *amhy* (Y chromosome-linked anti-Müllerian hormone) のホモログが単離された (Bej et al. 2017)。後代検定の結果から、*amhy* はギンイソイワシにおいても先に発見された南米原産トウゴロウイワシ目魚類 (Hattori et al., 2012、Yamamoto et al., 2014) と同様に Y 染色体上に存在することが示唆されており、本種では *amhy* 遺伝子を遺伝型性判別マーカーとして用いることで、遺伝型性 (XX or XY/YY) の判別が可能である (Bej et al., 2017)。従ってギンイソイワシでは、野生環境においてその性決定が水温の影響を受けているか否かを、雌雄比という集団レベルの観点からのみならず、性転換 (表現型性≠遺伝型性) という個体レベルの観点から調査可能であり、未だ全く明らかとなっていない日本近海に生息するトウゴロウイワシ目魚類の性の温度感受性解明の嚆矢となると期待される。

これまでの魚類の性へ水温影響に関する研究では、野生環境下での調査報告例は殆どない。その理由として、野生環境下では飼育環境とは異なり、水温や pH などの自然環境要因の変動のみならず、環境ホルモンなど人為的要因なども複雑に相互作用するため、例え雌雄の偏りや性転換が検出できたとしても、その現象と水温の因果関係を証明することが困難な点が挙げられる。本研究ではこの問題点を打開する為、耳石に着目した。耳石とは硬骨魚類の内耳に存在する炭酸カルシウムを主成分とした生体鉱物で、聴覚・平衡感覚をつかさどる器官である。顕微鏡下では、耳石断面に同心円状に明帯と暗帯が交互に形成された微細な輪紋構造が観察できる。日中には Ca に富む明帯が形成されるが、夜間には Ca の沈着は減少し代わってタン

パク質を主成分とする有機質の沈着が進行することで暗帯が形成される (Tanaka et al, 1981; Zhang and Runham, 1992)。多くの硬骨魚類で、この明帯と暗帯を 1 対とする輪紋構造が日周性を保持することが報告されており、現在は日齢査定や孵化日推定などに利用されている (Campana and Neilson 1985; Campana and Thorrold 2001; Pannella 1971)。また耳石は環境水中のストロンチウム (Sr) やナトリウム (Na) などの微量元素や同位体を取り込みながら成長し、代謝が著しく低いため、一度沈着した元素はほとんど変化しない。この耳石への微量元素の取り込みは、生息環境の水温や塩分などの外的要因に影響を受け、変化することが知られている (Campana 1999; Campana and Thorrold 2001)。したがって、水温変動と微量元素量変動の関係性が明らかになれば、性決定に水温は影響しているのか否かの因果関係の検証が可能になると期待される。

以上を背景に、本研究では日本沿岸域に生息する魚類の性に水温変動が与える影響調査を最終目的とし、第 1 章では日本近海に広く分布し、魚類でもっとも性の温度感受性が高いと考えられているトウゴロウイワシ目に属するギンイソイワシ *Hypoatherina tsurugae* の性決定への水温影響の調査を行った。第 2 章では野生個体の経験水温推定法の開発に向けて、ギンイソイワシ耳石内各種微量元素と水温変動の関連性の検証を行った。

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第 1 章

Study of thermal effect on sex determination of
cobaltcap silverside *Hypoatherina tsurugae*

1. Introduction

The temperature experienced during early development can affect sex determination and differentiation of the primordial, undifferentiated gonads in many fish species across phylogenetic groups (Baroiller, D’Cotta, & Saillant, 2009; Ospina-Álvarez & Piferrer, 2008; Strüssmann & Patiño, 1999). In the more thermally-sensitive species, which are considered as temperature-dependent sex-determined (TSD), a form of environmental sex determination (ESD), the population sex ratios are likely to vary greatly depending on the conditions of a particular breeding season. The worldwide trend of increasing temperature observed from the mid-20th century (Intergovernmental Panel on Climate Change, 2015), including in aquatic environments, has intensified concerns related to the impact of thermal abnormalities on the reproduction of such species (Ospina-Álvarez & Piferrer, 2008; Yamamoto, Hattori, Patiño, & Strüssmann, 2019). One of the groups with more examples of TSD species and that consequently deserves more attention is the Atheriniformes (Ospina-Álvarez & Piferrer, 2008; Strüssmann, Conover, Somoza, & Miranda, 2010; Yamamoto et al., 2019). At present, TSD has been documented in four genera of New World atheriniforms of the family Atherinopsidae: *Odontesthes* (South America), *Chirostoma* (Central America), *Leuresthes* (Central and North America), and *Menidia* (North America), but little is known about the eight families that inhabit the Old World (Brown, Baumann, & Conover, 2014; Conover & Kynard, 1981; Corona-Herrera et al., 2016; Middaugh & Hemmer, 1987; Strüssmann, Hanke, Cota, & Takashima, 1996; Strüssmann, Saito, Usui, Yamada, & Takashima, 1997; Yamamoto et al., 2019).

While in the past our group has devoted considerable efforts to determine the response of sex ratios to temperature and the physiological, molecular, and endocrine basis for TSD in atherinopsids through laboratory experiments, we have recently focused on the development of methods to analyze wild populations in order to obtain information on the

extent of TSD expression and its ecology directly from natural environments. Our approach comprises primarily of the development of genotypic sex markers, and if possible the elucidation of the sex determining gene itself (Bej, Miyoshi, Hattori, Strüssmann, & Yamamoto, 2017; Hattori et al., 2012; Koshimizu, Strüssmann, Okamoto, Fukuda, & Sakamoto, 2010; Yamamoto, Zhang, Sarida, Hattori, & Strüssmann, 2014) combined to otolith analysis (Strüssmann, Oikawa, Otake, & Kasuga, 2003). Although genotypic sex determination (GSD) and TSD were once considered to be incompatible (see Ospina-Álvarez & Piferrer, 2008), there seems to be little doubt now that both sex determination systems can coexist in the same species (Yamamoto et al., 2019, 2014). Thus, genotypic sex markers allow the detection of phenotypic/genotypic sex mismatches at the individual level and therefore provide more robust evidence of environmental influences on sex determination than could be obtained solely from phenotypic sex ratios. Otolith structural and chemical analyses, in turn, can provide the historical record of an individual including information on age (in years or days), estimation of the date of birth and past growth rates, and even of environmental exposure (Campana, 1999; Campana & Thorrold, 2001), that may help clarify the cause(s) for the sex mismatches.

In this study, we applied the molecular and otolith approaches to examine for the first time the effects of water temperature on the sex determination of an Old World atheriniform both in the laboratory and wild. The target of this study was the cobaltcap silverside *Hypoatherina tsurugae*, a marine atherinid with broad distribution and ecological importance as a forage species in coastal environments of Japan and South Korea (Mori, Kimura, Tsukamoto, Kawano, & Yoshida, 1988). In this species, we recently demonstrated the existence of a homologue of the male sex determining gene *amhy* of other atheriniforms (Y chromosome-linked anti-Müllerian hormone; Hattori et al., 2012, 2019; Yamamoto et al., 2014) and its close association with phenotypic sex in animals born and reared in captivity at

a constant temperature equivalent to the mean temperature during the spawning season (Bej et al., 2017). The primary objective of this study was to clarify if this genotypic sex determination can be overturned by high and low temperatures during gonadal sex differentiation. For this aim, we first examined the occurrence of phenotypic/genotypic sex mismatches in wild specimens from Tokyo Bay for 3 years (2014-2016) and used otolith analysis to estimate their birth dates and on-site logger-derived water temperature data to infer the approximate thermal history during the presumptive period of sex determination of each fish. In addition, the histological process of gonadal sex differentiation and the possibility of water temperature-dependent feminization and masculinization were examined in laboratory experiments with larvae reared under controlled thermal regimes.

2. Materials and Methods

2.1 Field survey of phenotypic/genotypic sex ratios and sex mismatches

This analysis was conducted in three different year-classes (2014, 2015, and 2016) of a population from Tokyo Bay. Cobaltcap silversides spawn in shallow areas and the young remain in coves near the spawning grounds through the juvenile stage until late autumn (Mori et al., 1988). Thus, sampling of juveniles from early to mid-autumn ensures unambiguous assignment to a particular year-class. Sampling was performed at night by hand net in a cove near the Tateyama Station, Field Science Center of Tokyo University of Marine Science and Technology (34.9N, 139.7E; Fig. 1). Fish were collected in October 2014 (n=338), November 2015 (n=244), and November 2016 (n=110). Samples were processed on spot and animals were treated as humanely as possible following the institutional guidelines for the care and management of laboratory animals and relief of pain. For this study, fish were anesthetized and subsequently killed in ice-water shortly after sampling. The standard length of each fish was measured, a small piece of the caudal fin was clipped and stored in 100 % EtOH for molecular analysis of genotypic sex, the gonads were excised and fixed in Bouin's solution for histological analysis of phenotypic (gonadal) sex, and the heads were stored at -20 °C for subsequent extraction and analysis of the otoliths.

The caudal fin tissue of each individual was treated in 90 µl of 50 mM NaOH at 95 °C for 10 min and equilibrated in 10 µl of 1M Tris-HCl buffer (pH.8.0). After centrifugation, the lysate supernatant containing genomic DNA was used as a template for sex genotyping based on amplification of the *amhy* gene (Bej et al., 2017). Amplification by PCR analysis was performed using the primers described previously (Bej et al., 2017) and a high-efficiency DNA polymerase (KOD Fx Neo, TOYOBO, Japan). PCR cycling conditions comprised 2 min at 94 °C, 40 cycles of 10 s at 98 °C, 30 s at 63 °C, and 1 min at 68 °C, and final elongation for 7 min at 68 °C. PCR products were electrophoresed in 1 % agarose gel

and visualized with ethidium bromide staining. Samples in which the *amhy* gene was amplified were considered as genotypic male (*amhy*⁺; note that by the current method it is not possible to distinguish XY and YY, henceforth XY/YY) and those in which it was not as genotypic female (*amhy*⁻; XX).

Samples fixed in Bouin's solution were dehydrated through an ascending ethanol series, cleared in xylene, embedded in Paraplast Plus (McCormick Scientific, St. Louis, MO), sectioned serially with a thickness of 4 µm, and stained with Mayer's hematoxylin and eosin. The phenotypic (gonadal) sex of each fish was determined by light microscopic examination following a previous study (Bej et al., 2017). Mismatches between genotypic and phenotypic sex (e.g. XX-testes/males and XY/YY-ovaries/females) were identified and used to calculate the occurrence of each type of sex transition per year-class.

2.2 Estimation of the birth dates and thermal history of normal and sex mismatching wild fish

Validation of the daily deposition of otolith increments was conducted using specimens from laboratory populations with known birth dates (see details of rearing in section 2.4). Briefly, fish were incubated from the eyed-egg stage through hatching and up to about 13 weeks after hatching at two temperatures, 22 and 26 °C. Three to four fish were sampled at 0, 16, 29, and 43 days after hatching (dah) and at 0, 14, 27, and 41 dah from the former and latter thermal regimes, respectively. Otolith increment analysis was used to estimate the hatching date of wild-captured juveniles. This analysis was first conducted on representative specimens that were chosen from the total catch so as to represent all size-classes from the smallest to the largest individuals (Fig. 2) and, assuming that body size and age are positively correlated, from the youngest to the oldest juveniles in each year-class. A

total of 56, 35, and 38 fish were chosen for the 2014, 2015, and 2016 year-classes, respectively, including both normal and phenotypic/genotypic sex mismatches. The sagittal otoliths were extracted from the heads, cleaned in distilled water, dried, and mounted in otolith grinding orientation blocks using UV-cured resin (Tama-Koubou, Kawasaki, Japan) (see details in Strüssmann, Miyoshi, & Mitsui, 2018). The otoliths were oriented in the blocks so as to allow grinding through the transversal plane and production of a thin cross-section including the otolith core that is already present at birth. Grinding was performed first from one side of the otolith block in a semi-automatic grinder/polisher (Doctor-lap ML-182; Maruto Instrument, Tokyo, Japan) fitted with a rotating specimen holder using increasingly finer abrasive paper (400-4000 grit; Reflex NAC M, Meiwafohis, Tokyo, Japan) when approaching to the otolith core (nucleus). Once near the core, the specimen was flipped and re-embedded in resin, and ground in a similar way from the opposite side. After the core was exposed, the resulting cross-section was fine-polished with diamond paste (DP-Paste; Struers, Copenhagen) on an automated polisher (Labopol-V, Struers, Copenhagen) to a mirror-finish, cleaned with deionized water, and dried. Sections were analyzed under a video microscope with transmitted light at magnifications of 100X–500X using the RATOC otolith measurement system (ARP/W+RI; Ratoc System Engineering, Tokyo, Japan). The number of daily increments from the core to the edge of each otolith was counted, subtracted by 6 to account for the number of increments formed before birth, and the resulting value was then subtracted from the collection date to estimate the birth date of each individual.

Estimation of the birth dates in the remaining fish whose otoliths could not be processed due to time and resource constraints was done using year-class-specific daily age/standard length relationships developed with the fish whose age was asserted through otolith analysis (Fig. 3). The birth dates of all fish sampled were then used to estimate the length of the breeding season and to examine the thermal history of individual fish from the

three year-classes during early development as follows. The water temperature profile in the environment where the individuals from each year-class supposedly spent from birth to the juvenile stage, as confirmed by periodic monitoring for the presence of larvae and juveniles, were inferred from temperature data recorded by a logger set in the study area (Fig. 1). Daily water temperature information from the logger was then used to calculate the average temperature experienced by each fish between hatching and the presumed time of gonadal sex determination, which was arbitrarily defined as the age in days when the fish reached an accumulated temperature of 300 °C based on the results of the histological analysis of laboratory-reared fish (see the next section and its results for details).

2.3 Histological process of gonadal sex differentiation and effects of water temperature on sex determination under laboratory conditions

The effects of water temperature on sex determination of cobaltcap silverside were tested in two rearing experiments with newly hatched larvae. The first experiment was also used to obtain information on the histological process and dynamics of gonadal sex differentiation in cobaltcap silverside. In the first experiment, wild, sexually mature adults (47 females and 5 males) collected by dip net were stocked to and allowed to breed naturally in a 500-liter tank supplied with filtered natural sea water at the Tateyama Station. Approximately 3,000 fertilized eggs were collected from the bottom of the tank in the following morning. In the second experiment, 28 females and 2 males were collected as described above and manually stripped in the evening to obtain ripe eggs and sperm for artificial fertilization. The gametes from all parents were pooled during artificial insemination, yielding approximately 3,000 fertilized eggs.

Fertilized eggs in both experiments were first incubated at 22 °C (Bej et al., 2017),

until the eyed-egg stage (6~7 days after fertilization). The eyed-egg embryos were then divided into three groups and incubated through hatching and up to 13 weeks after hatching (wah) at 18, 22, and 26 °C in the first experiment, and 22, 26, and 30 °C in the second. In both cases, the temperature was gradually adjusted within 12 hours after egg transfer. A different set of temperatures was used in the second experiment because of low hatching and survival rates at 18 °C in the first experiment.

Each aquarium had a volume of 60 L and was supplied with filtered, recirculated sea water at a flow of 50 ml/min. Aquaria were initially stocked with approximately 1,000 fertilized eggs and then with 300 newly hatched larvae. Dead eggs and larvae were removed every day. Larvae were fed rotifers *Branchionus rotundiformis* and *Artemia nauplii* to satiation twice daily from the first day after hatching and gradually weaned to marine fish food (Marubeni Nisshin Feed, Japan) from the third week.

Fish collected at 0, 2, 4, 6, and 8 wah (n=8–12) from the temperature of 22 °C in the first experiment and all fish alive at the end of both experiments (approximately 13 wah) were collected and processed for phenotypic and genotypic sex determination as described above.

2.4 Statistical analysis

The percentages of individuals with mismatching phenotypic/genotypic sex in different years in the field survey and at different temperatures in the rearing experiments were compared by the Fisher's Exact Test using GraphPad Prism (v.6.00; GraphPad Software, San Diego, CA, USA). Associations between the thermal experience of each fish and phenotypic sex in wild cobaltcap silverside of the 2014-2016 year-classes and in laboratory-

reared fish were assessed by Generalized Linear Modelling (GLM) using the statistical computing program R (R Core Team, 2018). Briefly, each fish was categorized as being phenotypic/genotypic sex-concordant or sex-mismatched and the explanatory variable was the average temperature during the estimated period of sex determination for each individual, either calculated as described above for wild fish or an actual measurement in the case of reared fish. The GLM analysis used a binomial error distribution and a probit link function and statistical significance was accepted at $p < 0.05$. Wild fish from the three year-classes were combined for this analysis but XY/YY and XX genotypes were analyzed separately in both field and laboratory studies.

3. Results

3.1 Field survey of phenotypic/genotypic sex mismatches

The *amhy*-based genotyping of wild-caught juveniles revealed balanced percentages of *amhy*⁺ (XY/YY) and *amhy*^{-/-} (XX) genotypes regardless of the year-class (Table 1). The histologically-determined phenotypic sex ratios, in turn, showed a gradual and significant increase in the percentage of males from year to year (e.g. 47.3, 58.6, and 78.2 % in 2014, 2015, and 2016, respectively). The percentages of phenotypic male/genotypic female (XX-males) also increased significantly year to year from 7.3 % in 2014, to 17.9 % in 2015, and 52.0 % in 2016. Those of phenotypic female/genotypic male (XY/YY-females), in turn, were similar in 2014 and 2015 (14.5 and 10.1, respectively), but significantly lower in 2016 (0 %). Thus, the 2014 and 2015 year-classes included both XX-males and XY/YY-females in addition to normal fish whereas in 2016 there were no XY/YY-females. Interestingly, the rates of sex transitions in either direction for each year showed a strong negative correlation ($R^2 = 0.995$; Fig. 4).

3.2 Birth dates and thermal history of normal and sex mismatching wild fish

The ranges of standard length of analyzed fish from 2014, 2015, and 2016 were 43.1-90.2 mm (mean \pm SD 74.5 \pm 10.2 mm), 35.5-84.6 mm (59.9 \pm 10.7 mm), and 32.0-78.1 mm (43.9 \pm 9.3 mm), respectively (Fig. 2). The determination of individual birth dates by otolith increment analysis revealed that the 2014, 2015, and 2016 year-classes were comprised of fish born between June 8 and August 20, July 13 and September 24, and August 13 and October 17, respectively (Fig. 5). The water temperature on these dates, and therefore in the beginning and end of the presumed breeding seasons, was 20.9 and 25.3, 21.2 and 24.2, and 26.7 and 24.0 °C, respectively. Sex mismatching XX-males were born almost throughout the

breeding season and therefore under varied thermal conditions in the three year-classes (Fig. 5). The XY/YY-females were born throughout the breeding season in 2014 and in the first half of the season in 2015 but were absent in 2016. Overall, sex mismatching XY/YY females either did not form or were few after the water temperature peaked in summer. The GLM analysis revealed that the likelihood of XX-male sex reversal was positively associated with the water temperature experienced prior to sex determination ($p < 0.01$) whereas for XY/YY fish no association could be demonstrated ($p > 0.05$) (Fig. 6).

3.3 Effects of water temperature on sex determination under laboratory conditions

The biweekly histological examination of fish reared at 22 °C revealed that cobaltcap silverside is a differentiated gonochoristic since gonads were found to develop directly from an undifferentiated primordium into ovaries or testes (Fig. 7). The first sign of gonadal sex differentiation at this temperature was the presence of prominent clusters of germ cells at 2 wah (accumulated temperature of ca. 300 °C), which indicated the beginning of ovarian formation in putative females as observed in other atheriniform species (Ito, Yamashita, Takashima, & Strüssmann, 2005). The putative testes at 2 wah remained histologically undistinguishable from undifferentiated gonads but at 4 wah there were clear signs of the formation of the main sperm duct (Fig. 7). All groups had balanced genotypic sex ratios regardless of the rearing temperature and experiment but variable rates of sex mismatching individuals at 13 wah (Table 2). As with wild fish (results not shown), the gonads of sex mismatching fish reared in the laboratory were histologically undistinguishable from those of sex matching, normal fish (Fig. 7). In the first experiment, the percentage of XY/YY-females was significantly higher at 18 °C (66.7 %) than at 22 °C (13.0 %) and 26 °C (8.3 %). The percentage of XX-males, in turn, was similar at 18 and 22 °C (16.7 and 10.3 %, respectively)

but significantly higher (47.4 %) at 26 °C. In the second experiment, the percentage of XY/YY-females showed no significant differences between temperatures (5.9, 0, and 5.6 % at 22, 26, and 30 °C, respectively) whereas that of XX-males increased significantly from 27.6 % at 22°C, to 52.8 % at 26°C, and 75.0 % at 30 °C. Replicates within each experiment were not available but the trends in phenotypic/genotypic sex mismatches at 22 and 26 °C in the first and second experiments were quite similar (Fisher's Exact Test, $p > 0.05$). In addition, the GLM analysis revealed highly significant, reciprocal associations between water temperature and the likelihood of sex reversal in both genotypes (Table 3). Thus, the proportions of XY/YY-females and XX-males were negatively and positively correlated with water temperature, respectively.

4. Discussion

This three-year field survey of young-of-the-year cobaltcap silversides from a wild population in Tokyo Bay revealed a progressive and significant departure from a balanced primary (phenotypic) sex ratio in 2014 (47.3 % male) to a mild and then marked male bias in 2015 (58.6 %) and 2016 (78.2 %), respectively. Biased sex ratios attributed to TSD have been reported before in studies with wild fish populations such as the cases of Atlantic silverside *Menidia menidia* (Conover & Kynard, 1981), the tidewater silverside *Menidia peninsulae* (Middaugh & Hemmer, 1987), and the southern flounder *Paralichthys lethostigma* (Honeycutt et al., 2019), but this study is unique in that the availability of a genotypic sex marker and the use of otolith analysis allow a detailed analysis of this phenomenon. First, it is noteworthy that none of the year-classes had significant deviations in the proportions of genotypic male (*amhy*⁺; XY/YY) and female (*amhy*⁻; XX), virtually ruling out a major contribution (albeit not a minor confounding effect) of differential mortality to the observed phenotypic sex bias. Second, the gradual male bias was strongly in phase with increasing frequencies of male-to-female transitions (XX-males; 7.3, 17.9, and 52.0 % in 2014, 2015, and 2016, respectively) and decreasing frequencies of female-to-male transitions (XY/YY-females; 14.5, 10.1, and 0 %) in the corresponding period. Finally, the rates of sex transitions in both directions were strongly negatively correlated, suggesting that the same factor(s) is(are) associated in both transitions but with reciprocal actions on female and male genotypes. This finding parallels observations in the rates of masculinization and feminization in another atheriniform, the pejerrey, a species known to have TSD (Yamamoto et al., 2014; Zhang et al., 2018). In that species, for example, an increase in temperature promotes female-to-male transitions while at the same time suppressing or making more difficult to occur male-to-female transitions. Overall, these results indicate that although the phenotypic sex often conforms to the genotypic sex, hence supporting the existence of a GSD

system and further validating the *amhy* gene as a sex marker/sex determinant in this species (Bej et al., 2017), it is also liable to environmental influences that likely include TSD and perhaps other environmental factors as well as discussed below.

The otolith analysis-based daily age and birth date estimation revealed that the three year-classes had breeding seasons of about the same length (e.g. approximately 2 months) but with a progressive delay of about one month per year between 2014 and 2016. These otolith-derived findings were confirmed not only by the observation of larvae during collection of juveniles, but also by observation of the presence of sexually mature adults near the experimental site (Miyoshi et al, unpublished observations). Generally, spawning shoals of cobaltcap silverside consisting of 1 to 3 years-old adults enter bay areas to shed gametes in shallow, vegetated areas (Mori et al., 1988; Tsukamoto & Kimura, 1993) between June and September in Central Japan (Okabe, 1993). The reason(s) for the progressive delay in the appearance of adults and spawning in this study are not known but could be related to particular conditions of ocean currents (e.g. Kuroshio current large meander) or stochastic climate phenomena such as typhoons. In fact, typhoons were recurrent during summer in 2016 (Japan Meteorological Agency, 2017). Notwithstanding the reasons, the delayed breeding season caused newborns to experience higher temperatures during early development from year to year which might help explain the observed year-to-year increases in the frequency of female-to-male sex reversal. Evidence supporting the assumption of temperature-modulated sex determination in cobaltcap silverside was obtained in the rearing experiments, which clearly demonstrated masculinizing and feminizing effects of high and low temperatures, respectively, and in the GLM analysis of the effects of water temperature experienced by wild fish between hatching and the critical time of sex determination, which indicated an increase in the frequency of female-to-male transitions with increasing temperature for the XX model. Interestingly, this study also uncovered compelling evidence

of the influence of daylength during early development on sex determination in cobaltcap silverside, with short and long daylengths inducing masculinization and feminization, respectively (Fig. 8). This places this species together with the grunion (Brown et al., 2014) and the pike silverside (Corona-Herrera et al., 2018), other atheriniforms in which sex determination is affected by photoperiod. Although this finding still needs to be scrutinized in carefully conducted laboratory experiments, including the evaluation of synergistic and antagonistic effects with temperature, in hindsight, it may help explain the seemingly paradoxical formation of sex-reversed males at the relatively low temperatures early and late in the breeding season, the inconsistency between results for wild and reared XY models, as well as the occurrence of some sex-reversed females at high temperatures in the laboratory experiments (which used a long, 14 hour photoperiod). However, an alternative explanation for these findings could be the presence of temperature insensitive genotypes or still several types of genomic or epigenomic alterations (see Baroiller & D’Cotta, 2016).

In any case, the above findings show that the cobaltcap silverside has TSD/ESD, or more specifically, the coexistence of genotypic and environmental sex determinants as previously demonstrated in an atherinopsid, the pejerrey (see Yamamoto et al., 2014, 2019). Some questions that still need to be addressed are 1) what would be the ecological implications of TSD/GSD for cobaltcap silverside, 2) the possibility of a latitudinal gradient in the strength of TSD as observed for the Atlantic silverside (Conover, 2004; Duffy, Hice, & Conover, 2015) and southern flounder *Paralichthys lethostigma* (Honeycutt et al., 2019) since the population we studied seems to be near the northernmost limit of distribution of this species (see Mori et al., 1988), 3) what are the climatic or biotic factors modulating the timing of the breeding season in this species, and finally, 4) what are the short- and long-term demographic implications of biased sex ratios and particularly of phenotype-genotype sex-mismatched individuals. Particular attention should be focused on the implications of

temperature-driven sex reversal for the existence of the putative Y chromosome (and hence GSD) demonstrated in this species (Bej et al., 2017). Field, laboratory, or theoretical studies in other species have predicted transgenerational effects of sex-reversed animals (Baroiller & D’Cotta, 2016; Cotton & Wedekind, 2009; Holleley, Sarre, O’Meally, & Georges, 2016; Wedekind, 2017). These studies suggested that if a sufficient number of such animals mature and reproduce, they could lead to the disappearance of one of the sex chromosomes and hence of genotypic sex determinants. Other considerations are how much genetic variance and potential for evolution exist in the reaction norms for thermal (environmental) sensitiveness of sex determination, and how fast this trait can evolve in cobaltcap silverside to match the new conditions imposed by climate change (see further discussions in Holleley et al., 2016; McGaugh & Janzen, 2011; Rhen, Schroeder, Sakata, Huang, & Crews, 2011a; Strüssmann et al., 2010; Wedekind, 2017). As noted above, we observed animals in both wild and laboratory experiments which seem to be either unresponsive to temperature (perhaps having a strong GSD?) or that show paradoxical sex reversal compared to the majority (for example, being feminized in otherwise masculinizing conditions). If these are signs of putative genetic variation in thermal sensitiveness as seen in other species (Baroiller, D’Cotta, Bezault, Wessels, & Hoerstgen-Schwark, 2009; Strüssmann et al., 2010; Wessels & Hörstgen-Schwark, 2007), they would certainly provide the genetic blueprint for evolution that might mitigate the negative effects of climate change. Even though we found a progressive increase in the frequency of males, it is still premature to predict large scale demographic problems with cobaltcap silverside for the reasons that this species appears to reproduce for at least 2 years, maybe 3 years, and because we have so far examined only one local population and for a limited period. Further studies should examine these issues which are crucial for the long-term stability of cobaltcap silverside populations.

Nevertheless, this study represents not only the first report of TSD in an Old World

atheriniform, but the first in a different family (Atherinidae) from all other atheriniforms in which TSD has been demonstrated so far (e.g. family Atherinopsidae). This point raises the obvious question as to whether TSD may be widespread, or even constitute an ancestral character in this Order (Strüssmann & Patiño, 1999). We are currently performing similar analysis in other atheriniforms that may provide answers to these questions. Assuming that TSD/GSD is a common character for all if not most atheriniforms and owing to their widespread distribution in coastal areas around the globe and gonadal sex thermolability, species in this group could constitute “early-warning” biological indicators of the effects of global warming and climate change on fish reproduction. We also would like to emphasize that the association of molecular and otolith analytical tools as employed in this study constitutes an extremely powerful approach for the systematic study of the effects of environmental factors on the sex ratios of wild fish populations.

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第2章

ギンイソイワシ耳石内各種微量元素と 水温変動の関連性の検証

1. 序論

第 1 章では耳石輪紋解析に基づく個体の推定孵化日と、捕獲地周辺に設置したロガーの水温データを基に野生ギンイソイワシ *Hypoatherina tsurugae* の経験水温履歴の推定を試みた。ギンイソイワシ仔稚魚は産卵期である初夏から晩秋にかけて、産卵場近辺の岩場や藻場を成育場として利用するため (Mori et al., 1988)、当歳魚の捕獲地周辺で記録された水温データは、性決定時期に仔稚魚が経験した水温を概ね反映していると考えられる。しかし一方で、前章の通りギンイソイワシは高水温では雄性転換(XX-雄)が、低水温では雌性転換(XY-雌)の出現率が上昇するが、フィールド調査の結果では、低水温期に孵化しているにもかかわらず雄性転換が、高水温期に孵化しているにもかかわらず雌性転換が僅かではあるが確認されている。これらの個体は、雌雄性分化に関わる遺伝子群に遺伝的な変異があり、遺伝的性とは逆の性に分化した可能性が考えられる(Shinomiya et al., 2004)。また、性決定時期の稚仔魚はすでに遊泳力を持つことから、適切な生息水温を求めて仔稚魚がロガーの測定地よりもより高温の浅瀬や、より低温の深場に移動し、他の個体と異なる生息環境を経験した結果、性転換が引き起こされた可能性も否定できない。安定した飼育条件下とは異なり、様々な要因が作用する野生環境下で生息する個体の調査を行うためには、単一のアプローチのみではなく、複数のアプローチをもとに、水温履歴推定の調査を行うことが求められる。

そこで第 2 章では、水温履歴推定法の一つとして、耳石の微量元素に着目した。

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2. 材料と方法

2.1 ギンイソイワシ飼育個体における水温と耳石内微量元素の関係性

以下の内容は、学術雑誌論文として出版する予定があるため公表できない
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2.2 野生ギンイソイワシにおける水温と耳石内微量元素の関係性

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3. 結果

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4. 考察

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総合考察

これまでに約 60 種の魚種で水温が性決定に影響を与えることが明らかとされているが、その多くは飼育環境下で調査されたものであり、野生環境での調査例はごく少数にとどまっている。その原因の一つとして、野生環境下では安定した飼育環境とは異なり、数多くある環境要因のなかから水温の影響を抽出し、その性決定との因果関係を証明することが困難であることが挙げられる。しかし、世界規模で観測されている地球温暖化・気候変動に起因した水温上昇によって魚類の性が影響を受ける可能性は高いと考えられ、野生環境における魚類の性と水温の関係を調査し、評価・監視することは極めて重要である。本博士論文研究では、*amhy* 遺伝子による性転換個体の検出、および耳石輪紋解析に基づく孵化日推定による経験水温履歴推定をもとに、野生および飼育環境下におけるギンイソイワシの性決定への水温影響の調査を行うとともに、野生個体の経験水温推定法の開発に向けて、ギンイソイワシ耳石内各種微量元素と水温変動の関連性の検証を行った。

第 1 章より、館山湾におけるギンイソイワシ当歳魚の捕獲調査を実施したところ、2014 年から 2016 年の間では、産卵(孵化)時期が年々遅れ、孵化直後の仔稚魚が年々高水温を経験したこと、その結果、遺伝型雌(XX)でありながら精巣を保持する性転換雄(XX-雄)が生じることで、雌雄比が雄に偏るという現象が生じていることが明らかとなった。高水温と性転換雄の出現率上昇の関係性は、飼育試験においても再現され、さらに一般化線形モデルにおいても支持された。以上より、本種は野生環境下においても飼育環境下と同様に高温での雄化が生じていることが明らかと

なった。野生環境下において、高水温に起因した雄性転換の増加を実証したのは本研究が世界初である。一方、雌性転換個体(XY-雌)も野生環境下および飼育環境下で確認された。飼育環境下における低水温と雌性転換の出現率の上昇の関係性は、一般化線形モデルにおいて支持されたが、野生環境下では両者の因果関係は実証できなかった。その理由として、調査期間中の生息地周辺の水温が比較的高温であったことに加え、野生環境下では飼育環境とは異なり、雌化を誘起する水温以外の要因が存在する可能性が考えられる。北米のトウゴロウイワシの仲間であるカルフォルニアグルニオンでは、短日条件下では雄の割合が、長日条件下では雌の割合が増加することが明らかとなっている(Brown, Baumann, & Conover, 2014)。そこで実際に、2014年から2016年に捕獲された野生ギンイソイワシ孵化日の日長時間と雌雄性転換の出現率の関係性を一般化線形モデルにより解析した結果、長日条件と雌性転換の出現率の上昇の関係性が支持された。したがって、本調査期間においては野生環境における雌性転換が、低水温条件ではなく、長日条件に起因して生じていた可能性が示唆された。また、短日条件と雄性転換の出現頻度の上昇の関係性も、一般化線形モデルにより支持された。本研究の調査では、2014年から2016年にかけて、孵化時期の水温は確かに年々上昇していたが、一方で、産卵期のずれが生じたことによって日長条件も年々短日化しており、複合的に雄への性転換に寄与していた可能性がある。今後、水温条件に加え、日長条件が本種の性決定に与える影響を飼育試験により併せて確認することで、両環境要因が本種の性に与える相互作用を調査していく必要がある。

先行研究では、北米大西洋沿岸に生息するヒラメの仲間である Southern flounder *Paralichthys lethostigma* において、米国ノースカロライナ南部に生息する地域集団において、2014 年から 2017 年の間継続して、水温に起因した性比の雄への偏りが生じていることが報告されている(Honeycutt et al., 2019)。また、アオウミガメ *Chelonia mydas* では、産卵巣の砂中温度が低温であると雄に、高温であると雌に性分化するが、近年、オーストラリア・グレートバリアリーフ北部で孵化した本種の性比は9割以上が雌に偏っていたことが報告された (Jensen et al., 2018)。さらに、この産卵場では過去 20 年以上、産卵期の砂中温度が雌化を誘起するほどの高温であったことが明らかとなっている (Jensen et al., 2018)。このように、温度依存型性決定機構を持つ種において、継続した生息環境の高温化に起因した極端な性比の偏りが確認されている。本博士論文研究では 3 年間にわたって当歳魚の野外調査を行ったが、地球温暖化や気候変動の影響が懸念される昨今、ギンイソイワシの産卵期においても、高水温が継続して生じる可能性は高く、今後も継続して野生環境下における水温変動とギンイソイワシの性比・性転換率の関係性を調査することで、これら影響の把握・評価を行っていくことが求められる。

第 2 章以下の内容は、学術雑誌論文として出版する予定があるため公表できない（5 年以内に出版予定）。

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