

Temperature-induced variation in sexual maturation of Japanese anchovy *Engraulis japonicus*

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*Temperature is one of the most influential factors for the sexual maturation of fishes, but understanding of the extent to which temperature affects the maturational schedules is limited in multiple-spawning fishes over a protracted season. This study examined the effect of temperature on sexual maturation of Japanese anchovy *Engraulis japonicus* siblings under high and low temperature regimes on different birthdates. The maturation probability differed between the two temperature regimes. Specimens in high temperature regimes matured at much smaller size and younger age than their counterparts. Also, a significant difference in the maturation probability between sexes was found at low temperatures, but not at high temperatures. Our findings show that temperature affects the maturational schedules of siblings of Japanese anchovy, suggesting that the size and age at sexual maturation could differ among cohorts, even in a given sampling location and/or year.*

Keywords: maturational decision, temperature, somatic growth, probabilistic maturation reaction norm, multiple spawning

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INTRODUCTION

Multiple batch spawnings of fishes promote their cohorts of offspring making it through the early stages of life and then surviving in various environmental conditions (Wright & Trippel, 2009; Lowerre-Barbieri *et al.*, 2011). This is typical for small and short-lived fishes like Clupeoids, in which females generally continue to produce a number of eggs in water with varying thermal conditions (Lambert *et al.*, 2003; Stratoudakis *et al.*, 2006). The timing of onset of sexual maturation is one of the key factors influencing fitness and recruitment success for spawning stock biomass, but this can be influenced by environmental and genetic factors (Heino & Dieckmann, 2008; Wright, 2013). Temperature is one of the most influential factors for the sexual maturation of fishes; it affects hormone synthesis and secretion, resulting in impacts on growth rate and gametogenesis (Lowerre-Barbieri *et al.*, 2011). Increasing temperature under *ad libitum* feeding conditions could lead to acceleration of somatic growth, resulting in the size threshold for a given species being reached at an earlier time and/or the investment of more energy in gamete production (Lambert *et al.*, 2003). On the other hand, temperature could directly affect the onset of sexual maturation without any improvement of growth conditions (Dhillon & Fox, 2004; Tobin & Wright, 2011), due possibly to the response of endocrine regulation

of gametogenesis to thermal conditions (Hermelink *et al.*, 2011). If the latter applies to fishes producing multiple batch eggs over a protracted season, the maturational schedule may differ among cohorts even in a given sampling location and/or year. However, our understanding of the extent to which temperature affects the maturation probability of such fishes is particularly limited.

Japanese anchovy *Engraulis japonicus* (Temminck & Schlegel, 1846) is a small pelagic species widely distributed around the waters off Japan. This species shows multiple batch spawning, mainly from spring to autumn with temperatures ranging from 15 to 28°C (Tsuruta & Hirose, 1989; Yoneda *et al.*, 2014). There is some evidence that the size at sexual maturation in Japanese anchovy differs markedly among sampling waters (Funamoto & Aoki, 2002; Funamoto *et al.*, 2004; Takasuka *et al.*, 2005; Sahara *et al.*, 2013). Funakoshi (1990) also reported that some of the small- and early-matured specimens were found in the collections of summer–autumn spawning fishes in the coastal waters off Japan. The causes of these differences, however, remain unexplored. As there are some cohorts successively recruited within the sampling waters in a given season (Funakoshi, 1990), it would be difficult to examine the timing of onset of sexual maturation of individual fishes from wild Japanese anchovy.

In this study, the timing of the onset of sexual maturation of Japanese anchovy was contrasted between high- and low-temperature regimes under *ad libitum* food availability. This species is a good model for investigation of the effects of the environment on reproductive traits under captive conditions (Tsuruta & Hirose, 1989; Kawaguchi *et al.* 1990; Yoneda *et al.*, 2013; 2014). To assess the maturational schedule, the

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probabilistic maturation reaction norm (PMRN) was used, in which the associations of the maturation process with age and size are accounted for by various sources of environmental and genetic variance (Heino & Dieckmann, 2008; Wright, 2013). This method models the probability of maturing as a function of age and size. As such maturity is estimated by cohort, making this approach appropriate for the investigation of maturation schedules, unlike maturity ogives which are confounded by the effect of varying juvenile growth and survival. Our main goal was to examine whether seasonal variation in temperature affects the maturational schedules of cohorts hatched out on different birthdates.

MATERIALS AND METHODS

Anchovies were caught in Hiuchi-Nada, in the middle of the Inland Sea, Western Japan, in June 2012, and were transferred to the Hakatajima station of the National Research Institute of Fisheries and Environment of Inland Sea. A total of 100 specimens were kept in a 2-ton circular tank at ambient temperature under a photoperiod cycle of 14 h light and 10 h dark to induce gonadal maturation (Tsuruta & Hirose, 1989). The water temperature in the tank was recorded every hour using a data logger (Tidbit V2, Onset, MA, USA). Fish were fed daily with 5–7% of their body weight (BW g) of commercial dry pellets (Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan, New Arteck: protein 52%, oil 11%, ash 18%, fibre 3%) per day.

Eggs were collected from the spawning fishes on 3 consecutive days in July (Exp. 1) and November (Exp. 2) 2012, for which spawning temperatures during the two periods were almost identical (22–23°C). A total of about 20,000 eggs (6000–7000 eggs per day collected) were kept in the 2-ton rearing tank. Thus, in this study, the day after hatching was defined as the first day of egg collection in each period. In Exp. 1, larvae and juveniles were reared at temperatures ranging from 26 to 28°C (mean 26.8°C; high temperature, HT), while those in Exp. 2 were kept in water of 20–22°C (mean 20.8°C; low temperature, LT) by using a hot water circulation pump system if necessary. During the two experiments, the siblings were reared under a photoperiod cycle of 14 h light and 10 h dark. S-type rotifers *Brachionus plicatilis* sp. complex cultured with highly unsaturated fatty acid-enriched *Chlorella vulgaris* (Super Chlorella V12; Chlorella Industry Co., Ltd, Fukuoka, Japan) were provided from age 2–40 days at a density of 20–25 rotifers ml⁻¹. The rotifer density was checked once daily and maintained by adding

new rotifers if necessary. *Artemia* nauplii enriched with highly unsaturated fatty acids (Super Capsule Powder; Chlorella Industry Co., Ltd, Fukuoka, Japan) were provided from age 30–50 days at a density of 0.2–1.5 *Artemia* ml⁻¹ and the commercial dry pellets (New Arteck) were also provided at 0.5–5.0% BW per day after age 40 days. Before the completion of metamorphosis (Fukuhara, 1983), 10 randomly collected fishes were measured to the nearest gram in BW every 5 days to check the amount of dry pellets provided. At age 50 days about 550 specimens at HT and about 600 specimens at LT were alive in the tanks of the two experiments. The filtered seawater was supplied from age 20 days to the end of experiments. The water exchange rate was kept at 30% of tank volume /day (25 l h⁻¹) at age 20–40 days, thereafter it was increased from 50 to 600% (42–500 l h⁻¹) over the course of the experiment. The ranges of dissolved oxygen and pH over the experiments were 5.8–7.0 mg l⁻¹ and 7.8–8.3, respectively.

Sample collections at HT and LT treatments started when all specimens had reached the juvenile stage at which metamorphosis had been completed (Fukuhara, 1983), and they were completed when >90% of mature specimens (see below) had been found. A total of 169 specimens at HT and 251 specimens at LT were collected. All specimens were killed by administering an overdose of 2-phenoxyethanol (600–800 ppm) in the morning on the day of sampling. Every 15 days, 35–94 randomly selected fishes were measured to the nearest millimetre in standard length (SL) and to the nearest gram in BW. The gonad (GW) and viscera weights (VW), including liver, gall and gas bladders, stomach, pyloric caecum, intestine and viscera fat weights, were determined to the nearest 0.1 g. Somatic weight (SW) was defined as:

$$SW = BW - (GW + VW)$$

The sample size was not fixed for each sampling period but it was adjusted to the expected maturation rate of the fish in which the data on fish sizes were consistent with a Gaussian distribution using the D'Agostino–Pearson normality test (Motulsky & Christopoulos, 2003). Small portions of the gonads were fixed in Bouin's solution for histological observation. After dehydration with ethanol, the gonadal tissues were embedded in methacrylate resin (Historesin, LKB). Methacrylate polymer resin sections 2 µm thick were stained with a 0.5% solution of toluidine blue. The testes and ovaries were classified into three stages of maturation, respectively (Table 1; Figure 1). No ovaries having migratory nucleus and hydrated stages were collected as final oocyte maturation

Table 1. Description of microscopic characteristics of testes and ovaries of Japanese anchovy *Engraulis japonicus* at different stages of maturation.

Stage	Characteristics
Males	
Immature	Germinal cysts containing spermatogonia and spermatocytes were observed. No spermatozoa were found in the lumina of the lobules (Figure 1A)
Spermatogenesis	Germ cells at all stages of spermatogenesis were present and spermatozoa were also found in the lumina of lobules (Figure 1B)
Mature	Large quantities of spermatozoa were present in the lumina of lobules (Figure 1C)
Females	
Immature	Only previtellogenic oocytes (pre-nucleolus and cortical alveoli stages) were found (Figure 1D)
Early vitellogenesis	The most advanced oocytes had reached early yolk stage (Figure 1E)
Late vitellogenesis	The most advanced oocytes had reached late yolk stage (Figure 1F)

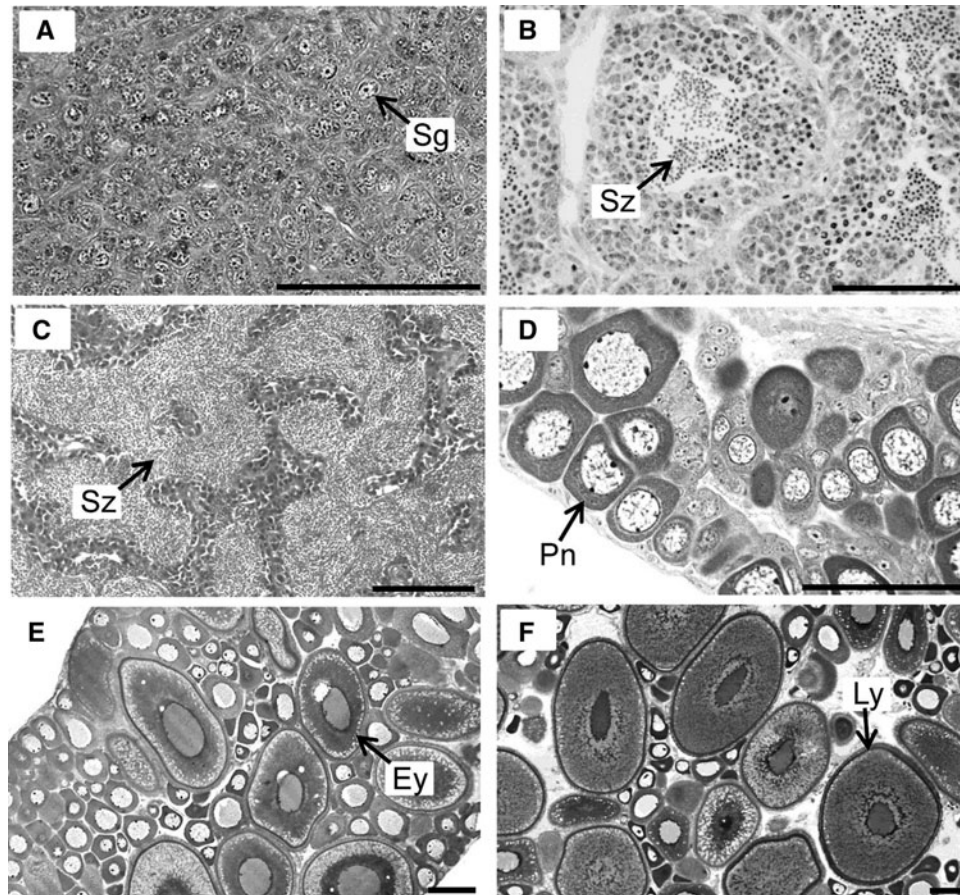


Fig. 1. Microscopic characteristics of testes and ovaries of Japanese anchovy *Engraulis japonicus* at different stages of maturation. (A): immature stage of testis; (B): spermatogenesis stage of testis; (C): mature stage of testis; (D): immature stage of ovary; (E): early vitellogenic stage of ovary; (F): late vitellogenic stage of ovary. Ey, early yolk stage oocyte; Ly, late yolk stage oocyte; Pn, perinucleolus stage oocyte; Sg, spermatogonium; Sz, spermatozoa. Scale bars = 100 μm .

proceeds from afternoon to evening (Yoneda *et al.*, 2013). Mature specimens were defined as testes at spermatogenesis or mature stages for males and ovaries at early and late vitellogenic stages for females. In the PMRN analysis, only specimens having spermatogenesis testes and early vitellogenic ovaries were used to ensure the size and age at the onset of sexual maturation (Heino & Dieckmann, 2008). To estimate PMRNs, the direct estimation method was used; numbers of immature and newly mature individuals sharing a certain combination of age and size can be seen as resulting from independent realizations of the probabilistic maturation process.

Statistical analyses were performed with the software R version 3.0.2 (R Development Core Team, 2013). To test the difference in the *SW-SL* relationship between HT and LT treatments, a generalized linear model (GLM) incorporating a Gaussian response distribution coupled with log link function to connect the mean to the linear predictors was used. Additionally, to determine the effect of *SL* or *SW*, age class and sex on maturation, GLM incorporating a binomial response distribution coupled with logit link function was used. Age class was classified into five groups at 15-day intervals between age 60 and 120 days, defined as a categorical factor. In this analysis, the GLM was run separately in each treatment for maturation. Explanatory variables were assessed for possible collinearity visually and using variance inflation factors (VIF) in the package DAAG. All models had VIF < 2.0. The model fittings were conducted using stepwise

backward selections based on the Akaike Information Criterion:

$$\text{AIC} = 2k - 2\ln(L)$$

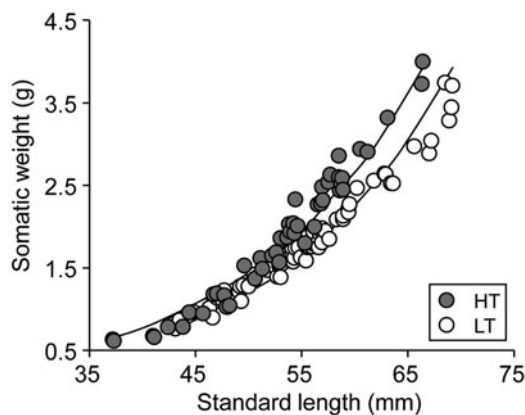
where k represents the number of parameters in the fitted model and L represents the maximized value of the likelihood. A chi-square test was also used for analysis of deviance. A PMRN were visualized by a collection of curves following isoprobability contours. As such, the midpoints of a PMRN pass through all sizes for which maturation occurs with a probability of 50% and the width of a PMRN for each age shows the *SW* interval at which the probability of maturation rises from 25 to 75%.

RESULTS

The per cent of mature specimens collected at given age classes differed between LT and HT (Table 2). At HT, 50% (13/26) and 40% (14/35) of males and females at age 60 days were mature and about 90% of specimens had reached maturation at age 75 days. At 90 days, all specimens at HT had either mature testes or late vitellogenic ovaries. At LT, no mature specimens were found at age 75 days, but thereafter, the per cent of maturation of both sexes increased. Most of the specimens at age 120 days had become mature.

Table 2. Number of mature specimens (M) of Japanese anchovy *Engraulis japonicus* at different age classes under low (LT) and high (HT) temperature treatments.

Age class	LT				HT			
	Males		Females		Males		Females	
	M	Total	M	Total	M	Total	M	Total
60 days					13	26	14	35
75 days	0	32	0	62	25	28	39	45
90 days	14	25	15	37	21	21	14	14
105 days	28	29	21	31				
120 days	19	19	15	16				

**Fig. 2.** Relationship between somatic weight and standard length of Japanese anchovy *Engraulis japonicus* at high (HT, closed circles) and low (LT, open circles) temperature regimes at age 75 days. Only specimens having gonads with early vitellogenesis and spermatogenesis stages were used.

In the specimens having gonads without late vitellogenesis or mature stages at age 75 days, initial GLM showed no significant difference in the SW and SL regression between the sexes ($\chi^2 = 1.72$, $P = 0.19$); thus, the data were combined. There was a significant difference in the regression of the SW-SL relationship between HT and LT (HT: $N = 53$, LT: $N = 67$, $\chi^2 = 81.6$, $P < 0.001$, Figure 2). Specimens at a given SL at HT had about 16% heavier SW than those at LT, indicating that individuals were growing faster at HT.

The stepwise backward selections indicated that the effect of SL or SW and age class on maturation were significant at HT while that of SL or SW and sex was significant at LT. However, the GLMs incorporating SW and age class or sex showed lower AICs at HT (58.8) and LT (97.4) (Table 3) than those including SL and age class or sex at HT (59.8) and LT (103.7); thus the former models were selected. The midpoints of SW of maturation probability at given ages differed between HT and LT (Figure 3), indicating that specimens at HT matured at a much smaller size and younger age than those at LT. The response of the maturation probability to thermal conditions between sexes also differed: At LT, the effect of sex on the probability was significant ($\chi^2 = 6.70$, $P < 0.01$), but not at HT ($\chi^2 = 1.06$, $P = 0.30$). At LT, males on given days from birth had matured at a smaller size than females.

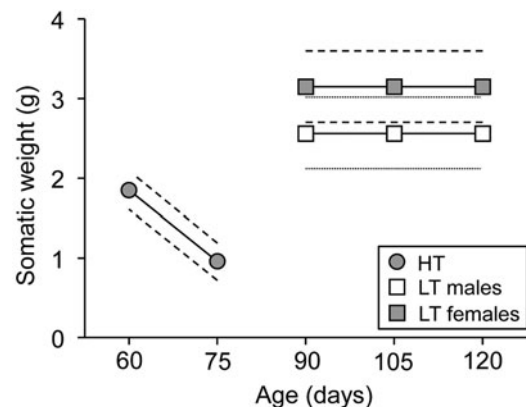
DISCUSSION

Our findings showed that temperature has a marked effect on the maturational schedules of siblings of Japanese anchovy

Table 3. Summary table of parameter estimates and analysis of deviance for generalized linear models (bi-nominal family and logit link) for sexual maturation of Japanese anchovy *Engraulis japonicus* at high (HT) and low (LT) temperature treatments. Explanatory variables in the models selected based on the Akaike information criterion (AIC) are shown. Somatic weight (SW), age class (A) and sex were used as initial variables in each treatment. The explained deviance of the model (ED) is shown.

Model summary	Estimate	SE	d.f.	Deviance	P
HT (ED = 62%, AIC = 58.7)					
Intercept (Null)	-8.44	1.76	98	137.8	
SW	-4.56	0.96	1	53.8	<0.001
A 75 days	4.07	0.99	1	31.3	<0.001
LT (ED = 43%, AIC = 97.4)					
Intercept (Null)	-6.25	1.23	119	161.5	
SW	2.44	0.46	1	63.4	<0.001
Sex	-1.45	0.58	1	6.7	<0.01

reared under identical photoperiod cycles. Most specimens became mature by age 75 days at HT, while males and females at LT started maturation after age 75 days. A significant difference in SW-SL regression at age 75 days showed more rapidly growing specimens at HT than at LT, but the PMRN estimations indicated that specimens at HT matured at much smaller SW on given days of age examined than those at LT. This implies that the temperature specimens have experienced

**Fig. 3.** Probabilistic maturation reaction norms (PMRN) of Japanese anchovy *Engraulis japonicus* with 50% quantiles (midpoints) estimated for age- and somatic weight-based PMRN models at high (HT, circles) and low (LT, squares) temperature treatments. Dotted lines indicated 25 and 75% quantiles. The midpoints of a PMRN pass through all sizes for which maturation occurs with a probability of 50% and the width of a PMRN for each age shows the somatic weight interval at which the probability of maturation rises from 25 to 75%.

during larval and juvenile stages may change the somatic size threshold for the onset of sexual maturation. These observations appear to be consistent with the well-established general prediction that ectotherms mature earlier at a smaller size when the growth rate is stimulated by increased temperature (Berrigan & Charnov, 1994). In addition, we did not rule out the possibility that temperature directly affects the onset of sexual maturation, possibly via a physiological process associated with endocrine changes (Tobin & Wright, 2011). The rate of oocyte growth of Japanese anchovy is markedly influenced by prevailing temperatures, resulting in temperature-dependent variation in batch interval (Yoneda *et al.*, 2014).

Our findings show a difference in the response of the maturation probability to thermal conditions between the sexes. The effect of sex on the probability was significant at LT, with males at a given day of age reaching a smaller size at sexual maturation than females. This may in part have been caused by a sex-related difference in the rate of energy accumulation at the critical period of maturational decision, as males generally utilize less energy for gonadal maturation than females (Wootton, 1990). However, this assumption does not appear to match the observations at HT, at which there was no significant difference in the maturation probability between the sexes. If the downward shift in the energetic threshold for maturation occurs at HT, more rapid growth might diminish the occurrence of a sex-dependent difference in the maturational probability, at least in our sampling periodicity (15-day intervals). In pikeperch *Sander lucioperca* (Linnaeus, 1758), warmer water within the range of species-specific spawning temperatures promoted puberty of both sexes through increasing levels of gonadotropin and sex steroid hormone (Hermelink *et al.*, 2011).

Our findings show that temperature markedly affects the maturational schedules of siblings of Japanese anchovy. It would be reasonable to assume that the size and age at sexual maturation could differ among cohorts, even in a given sampling location and/or year. The spatial variation in size at sexual maturation found in Japanese anchovy, therefore, may in part be caused by the temperature fishes have experienced in given waters (Funakoshi, 1990; Funamoto & Aoki, 2002; Takasuka *et al.*, 2005; Suhara *et al.*, 2013). These observations may be relevant in short-lived fish species producing multiple batch eggs over a prolonged season, especially in Clupeoids, and caution should be taken when estimating the stock reproductive potential of such fish species by considering the temperature-related maturation probability (Wright, 2013).

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