

Temperature- and income resource availability-mediated variation in reproductive investment in a multiple-batch-spawning Japanese anchovy

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ABSTRACT: Multiple batch spawning over a season should have an advantage for maternal fitness in unpredictable environments, but the manner in which females allocate resources to reproduction is not well understood. We explored the effects of temperature and food availability on reproductive traits in Japanese anchovy *Engraulis japonicus* using several physiological analyses. Under ad libitum food availability, temperature had a significant effect on batch intervals by changing the growth rate and size of oocytes during vitellogenesis. The latter is likely to result in variations in the size and total nutritional content of eggs. Relative batch fecundity, however, was not significantly influenced by temperature regimes. Reproductive effort per spawning was significantly higher at lower temperature, caused by the production of eggs with higher nutritional content, while total reproductive effort in a given period of time increased in water at a higher temperature due to shortening of batch intervals. Short-term food manipulation also had a significant effect on reproductive output. Insufficient income resources could lead to prolonged batch intervals, but the relative batch fecundity remained constant independent of the temperature regime. Our findings suggest that female Japanese anchovy may maintain reproductive effort per spawning relative to the prevailing temperature at the expense of more spawning events in a nutritionally harsh environment. Thus, although the total reproductive effort of well-fed specimens increased in water with increasing temperature due to shortened batch intervals, this variation may be caused by a compensatory response to the level of income resources available.

KEY WORDS: *Engraulis japonicus* · Phenotypic plasticity · Energy allocation · Reproductive effort · Batch interval · Egg size · Egg production

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INTRODUCTION

Life history theory predicts that organisms will partition surplus energy among growth, reproduction, and storage in a way that maximizes their lifetime reproductive success (Roff 1992, Stearns 1992).

Given that reproduction is a costly process in which resources are temporarily sacrificed or traded off from other life functions, how individuals allocate their resources in terms of offspring number and size has been one of the key issues in research of life history theory and population dynamics (Duarte &

Alcaraz 1989, Stearns 1992, Einum & Fleming 2000). Numerous aquatic organisms, such as fish and invertebrates, exhibit multiple batch spawning over a breeding season, which may increase the probability of larval survival by enhancing the likelihood of encountering favorable environmental conditions (Leggett & Frank 2008, Wright & Trippel 2009). Thus, to maximize her fitness, a female may need to optimize not only the number and size of eggs produced within a single spawning, but also her total productivity per season (Roff 1992, Wootton & Fletcher 2009). However, our understanding of energy allocation to reproductive output over that season is particularly limited in fish, since temporal and spatial variations in environmental factors can have profound effects on the energy allocation strategy of individuals (Lambert et al. 2003, McBride et al. 2013).

Multiple batch spawning in many fish species can largely depend on the energy acquired during the ongoing spawning season, which is called income breeding (McBride et al. 2013). This contrasts with the previous theory that ectothermic organisms may largely be capital breeders, for which the current reproductive output is primarily a function of energy accumulated over long periods prior to reproduction (Bonnet et al. 1998). Income breeding has the advantage of fine-tuning reproductive output relative to the current environment in terms of both maternal condition and potential survival of the offspring. Although field and laboratory experiments using fish exhibiting multiple batch spawning show a significant relationship between prey abundance and/or food rations and egg production in a given period of time (e.g. Tsuruta & Hirose 1989, Peebles 2002, Somarakis et al. 2012), little attention has been paid to the interaction between temperature and food intake on reproductive investment. As changes in food intake and metabolic rate are associated with a change in temperature, an unfavorable temperature could reduce energy investment in growth and reproduction by a reduction in food consumption and/or increases in metabolic and activity costs (Jobling 1993, Pörtner et al. 2006). Therefore, the optimal allocation of energy to reproductive outputs for such species may change under different thermal regimes, even if food availability remains constant.

Egg size is among the most important surrogate measures of maternal investment and is linked to a number of fundamental and adaptive traits of offspring (Chambers 1997, Moran & McAlister 2009). In fish and invertebrates, for which released eggs disperse in the water column, egg size exhibits temperature-related variation, with larger eggs produced in

cooler water. This phenomenon may be due to a direct physiological response in which the temperature experienced by a female during oogenesis influences the size of eggs she produces (Laptikhovskiy 2006, Moran & McAlister 2009). Thus, egg size is not necessarily a selected trait as part of a reproductive strategy, but its association with egg number cannot be ignored (Marshall et al. 2008). Given that egg size is physiologically related to the prevailing temperature, variation in batch interval (BI) may also be caused by physiological responses to thermal conditions. This potential mechanism of BI could include different temperature constraints on oocyte recruitment, rate of vitellogenesis, and de novo vitellogenesis (Lowerre-Barbieri et al. 2011, Migaud et al. 2013). It may be that the increased egg size at low temperature is caused by a deceleration of physiological processes, resulting in reduced growth rates and extended development times of oocytes in the ovary. These factors are likely to be accompanied by prolonged BIs in cooler water.

The assumption is that, under certain thermal conditions with a favorable level of available prey, the amount of resources that a female can convert to eggs should directly determine her reproductive output. Conversely, if resource availability is limited at a given temperature, the physiological process of converting resources to eggs may be limited, possibly accompanied by variations in egg size, batch fecundity, and/or BIs. Such variations may be a function of the type of nutritional competition, along with the degree of plasticity vs. genetic constraints on resource acquisition and allocation (Boggs 2009). Clearly, any analysis of resource availability-mediated changes in reproductive traits cannot be performed without considering temperature-related differences.

The purpose of this study was to explore the effects of temperature and food availability on reproductive traits of Japanese anchovy *Engraulis japonicus* using several physiological analyses. This species is a good model for investigating the effects of the environment on reproductive traits under captive conditions (Tsuruta & Hirose 1989, Kawaguchi et al. 1990, Yoneda et al. 2013). Japanese anchovy exhibits multiple batch spawning over a prolonged spawning season and is believed largely to be an income breeder. Thus, rather than regulation of the degree of food availability over a long period, short-term food manipulation could be more beneficial to understand the reproductive allocation strategy of this species. We first examined the effect of temperature on reproductive traits under different temperature regimes within the range of natural variation experienced by wild fish

during a spawning season. As temperature largely influences food intake (Jobling 1993), specimens provided with feed ad libitum were used in this analysis. To examine the physiological aspects of egg production of individuals, the growth rate and size of oocytes were monitored using ovarian biopsy and *in vitro* assays (Yoneda et al. 2013). As the size of eggs produced is not necessarily an accurate indicator of their energy content (Moran & McAlister 2009), the relationships between the amounts of elemental carbon (C) and nitrogen (N) per egg and temperature were also examined. Next, the effect of short-term food availability on reproductive effort was compared among 3 feeding regimes and between 2 different temperature regimes. We addressed the following 3 questions: (1) How do females adjust particular reproductive traits under a given set of thermal conditions? (2) Do females change their reproductive effort in successive batches over a season? (3) Are some traits preserved at the expense of others, or are all trait values suppressed in a nutritionally harsh environment?

MATERIALS AND METHODS

Experimental set-up

Anchovies were caught in Ohmura Bay, Nagasaki Prefecture, Japan, and maintained for several weeks in the sea cages of a fishing company (Takeshita Suisan, Nagasaki, Japan). Specimens in these sea cages were transferred to the Fishery Research Laboratory of Kyushu University, Fukuoka Prefecture, in March 2009 and February 2010 and 2011. The fish were kept in circular tanks (1000 and 3000 l) at ambient temperature and under a natural photoperiod cycle. The water temperature in each tank was recorded every hour using a data logger (Tidbit V2). Fish were fed daily with 5 to 7% of body weight (BW, g) of commercial dry pellets (Marubeni Nisshin Feed; New Arteck K-4: protein 52%, oil 11%, ash 18%, fiber 3%) 6 times d⁻¹. The initial stocking density in all tanks was ca. 0.075 ind. l⁻¹.

Specimens were measured to the nearest millimeter for body length (BL), to the nearest gram for BW, and to the nearest 0.1 g for ovarian weight. BL is the length of a fish measured from the tip of the snout to the posterior end of the midlateral portion of the scale. Ovarian pieces were fixed with 10% neutral buffered formalin for oocyte measurement and with Bouin's solution for histological observation. Condition factor (CF) was expressed as follows (see Table 1 for a summary of abbreviations used in this study):

Table 1. Abbreviations (Abbr.) of reproductive parameters used in this study

Abbr.	Meaning
BI	Batch interval in water of a given temperature; calculated from Eq. (4)
CF	Condition factor; calculated from Eq. (1)
EV	Egg volume; calculated from Eq. (3)
OGR	Oocyte growth rate during vitellogenesis; calculated from Eq. (2)
RBF	Relative batch fecundity; calculated from Eq. (5)
RE	Reproductive effort per spawning; calculated from Eq. (8)
REP	Relative egg production on the day of the experiment in each tank; calculated from Eq. (9)
TRE	Total reproductive effort at a given period of time; calculated from Eq. (7)
VE	Mean volume of vitellogenic oocytes collected at 00:00 h
VL	Mean volume of vitellogenic oocytes at a given sampling hour

$$CF = \frac{OFW \times 10^5}{BL^3} \quad (1)$$

where ovary-free weight (OFW, g) was calculated as BW – ovarian weight. Mean BLs and CFs of specimens on the day of onset of rearing in 2009 to 2011 were 88–94 mm and 0.83–0.95, and most (88–100%) individuals were immature. As time passed, CF and the maturation percentage gradually increased. Daily spawning in each tank occurred in water >15°C (Tsuruta & Hirose 1989). Mean BLs and CFs of females at the onset of spawning (late April to early May) were 93–95 mm and 1.15–1.22. Variation in CF of specimens was not size dependent, as there was no significant relationship between BL and CF of females under ad libitum feeding conditions (n = 713, r² = 0.0003, p = 0.80).

Effect of temperature on reproductive traits

In Japanese anchovy, the duration from the completion of vitellogenesis (late vitellogenic stage) to the onset of spawning appeared to be within about 24 h, and the most advanced oocytes in the ovaries just after spawning were at the early vitellogenic stage (Yoneda et al. 2013). Thus, it is assumed that the duration from the early stage to late vitellogenesis could be the most influential factor to estimate the BI of individuals. To examine the relationship between BI and temperature, 4 parameters, namely, oocyte volumes at early and late vitellogenesis (VE

and VL, respectively), oocyte growth rate (OGR) during vitellogenesis, and mean daily spawning time, were estimated. The estimation of VE was based only on ovaries with newly postovulatory follicles collected just after spawning, while VL was estimated using ovaries showing a positive response in an *in vitro* assay (Yoneda et al. 2013). To examine OGR, ovarian tissues of individual females were repeatedly collected by ovarian biopsy. After anesthetizing the fish with 2-phenoxyethanol (200 ppm) at 00:00 h, a gonadal biopsy using a plastic catheter (1.5 mm internal diameter) was taken to determine sex. The collected ovarian tissue was kept in 10% neutral buffered formalin for the measurement of oocyte size and was then placed in Sera solution (ethanol:formalin:acetic acid = 6:3:1) for rapid identification of the oocyte developmental stage, in addition to immersion in Bouin's solution for histological observation. For only females with vitellogenic oocytes, an elastomer fluorescent tag (Northwest Marine Technology) was individually injected around the adipose eyelid under anesthesia, and specimens were then transferred into a 0.5 t circular tank at a density of 50 ind. t⁻¹ and a 1:1 sex ratio. At 12 to 23 h after the collection of the ovarian biopsy, the marked females were sacrificed and the ovary was preserved in 10% formalin and Bouin's solution. OGR of individual females was calculated as follows (Table 1):

$$\text{OGR} = \frac{\text{VL} - \text{VB}}{\text{L}} \quad (2)$$

where VE is the mean volume of vitellogenic oocytes collected at 00:00 h and VL represents the mean volume of vitellogenic oocytes at a given sampling hour (L), i.e. the time passed (in h) since 00:00 h. Because anchovy oocytes are ellipsoid, both long and short axes were measured for the 70 to 150 largest oocytes, using Image-Pro Plus ver. 5 (Media Cybernetics). Oocyte volume (mm³) was calculated as follows.

$$\text{Volume} = \frac{\text{long} \times \text{short}^2 \times \pi}{6} \quad (3)$$

In each ovary, we only measured oocytes with volume >0.017 mm³, that is, the minimum size of vitellogenic oocytes found in the ovaries with newly postovulatory follicles just after spawning. To normalize the size-frequency distribution of the most advanced oocytes in the ovaries, a Gaussian distribution model was fitted to the observed data on oocyte volume in each ovary after the data had been shown to be consistent with a Gaussian distribution using the D'Agostino-Pearson normality test (Motulsky & Christopoulos 2003). The estimated mean oocyte volume was used in the relevant analyses. For the estimation of egg volume (EV) on a given day of sampling, this method of oocyte

measurement was also followed. In the analysis of the relationship between EV and temperature, mean daily temperature in the last 2 d was used during the period when oocytes had proceeded from late vitellogenesis to ovulation, including the process of final oocyte maturation (Yoneda et al. 2013).

The mean daily spawning time during the experiment was estimated from the relative egg production rate (Yoneda et al. 2013), and models of the relationships between relative egg production rate and temperature and the estimated mean daily spawning times were expressed (see Tables S1 & S2 in the Supplement at www.int-res.com/articles/suppl/m516p251_supp.pdf).

The BI (days) in water of a given temperature was calculated as follows:

$$\text{BI} = \frac{\text{DV} + \text{DF}}{24} \quad (4)$$

where DV is expected duration (hours) from early to late vitellogenesis at a given temperature, and DF is expected duration from midnight (00:00 h) to the expected mean daily spawning time at a given temperature. These expected parameters were derived from the final model of the relationship between the relevant trait and temperature (see 'Results'). This concept of BI is similar to that of Atlantic sardine *Sardina pilchardus* (Ganias et al. 2011).

To determine the energy content of an egg to be spawned, the amounts of C and N per egg were examined using only specimens reared between April and July 2011. Eggs were thawed at room temperature and rinsed with distilled water; thereafter, egg samples were counted under a microscope and freeze-dried in a micro-tube. About 300 to 1000 eggs were pooled for each egg sample to ensure sufficient numbers for the analysis. Dried samples were ground to a fine powder and placed in tin capsules. In order to assess the whole energy in the eggs, lipids were not removed. The analysis of total C and N was conducted using an elemental analyzer (Flash EA, Thermo Electron). The amount of C or N of an egg was estimated from the total amount of C or N divided by the total number of eggs used. In this analysis, eggs were collected from specimens reared under 3 types of food manipulation using 2 different sources of commercial dry pellets (New Arteck and Torafugu EP: protein 53%, oil 8%, ash 15%, fiber 3%). Of these, in 2 manipulations, 1 type of the dry pellets was provided throughout the experiment. The other involved a switch of diet, where New Arteck was initially provided but then switched to Torafugu EP. All fish were fed to satiation with these 2 types of dry pellet feed.

Batch fecundity was estimated from specimens with ovaries containing migratory-nucleus and hydrated-stage oocytes using a gravimetric method (Yoneda et al. 2013). Females that had ovulated oocytes were excluded from this analysis. Samples (0.2–0.5 g) were collected from the central parts of the left ovary. Batch fecundity for each female was calculated as the number of migratory-nucleus and hydrated-stage oocytes per unit weight, multiplied by the ovarian weight. During the experiment, spawning females gradually grew as the season progressed. To remove the effect of the timing of collection on body size, the relative batch fecundity (RBF) was calculated as follows:

$$\text{RBF} = \frac{\text{batch fecundity}}{\text{expected OFW}} \quad (5)$$

where expected OFW was calculated from all females ($n = 713$, $r^2 = 0.63$) fed to satiation between May and August, expressed as follows.

$$\text{Expected OFW} = 6.7 \times 10^{-5} \times \text{BL}^{2.62} \quad (6)$$

Total reproductive effort (TRE) at a given period of time was calculated as follows:

$$\text{TRE} = \frac{\text{RE}}{\text{BI}} \quad (7)$$

where RE is reproductive effort per spawning expressed below.

$$\text{RE} = \text{RBF} \times \text{CWT} \quad (8)$$

Here, CWT is the expected amount of C per egg calculated from the equation model of the relationship between the amount of C and temperature (see 'Results'). In these analyses, specimens were collected during the 3 periods subjected to different temperature regimes: 20°C (in May), 24°C (in June–July), and 28°C (in July–August).

Effect of food availability on reproductive traits

Food manipulation experiments were conducted during the 2 periods of different temperature regimes in May (Expt 1) and July 2009 (Expt 2). All specimens were reared in a 3 t circular tank before the onset of the experiment. After measurement of the fish body condition of sub-samples, 50 randomly selected specimens were transferred into 6 circular tanks (1 t) at ambient temperature and under a natural photoperiod cycle. Prior to the food manipulation, daily spawning was monitored for 7 d under satiation (5% BW d⁻¹) to estimate the mean total number of eggs produced daily in each tank. At the onset of food manipulation, specimens were subjected to 1 of the 3

feeding regimes: high (HF, 5% BW d⁻¹), low (LF, 1.75% BW d⁻¹), or starvation (ST, 0% BW d⁻¹). Duplicates of each feeding regime were performed. The total number of eggs produced in each tank was monitored every day, and the EV was also measured daily after fixation in 10% neutral formalin. All specimens were sacrificed on Day 14 of the experiment to examine the CF of individuals and the RBF in the HF and LF treatments. Only 4 and 2 specimens having migratory-nucleus or hydrated-stage oocytes in the ST treatment were collected at the end of Expts 1 and 2, thus the estimate of RBF in ST was excluded. Temperature in Expt 1 was 19.5°C (range: 18.9–20.1°C), while that in Expt 2 was 23.7°C (range: 23.1–24.2°C).

To determine the effects of food availability on reproductive traits, the relative egg production (REP) on the day of the experiment in each tank was calculated (Table 1):

$$\text{REP} = \frac{\text{TE}}{\text{TEB}} \quad (9)$$

where TE is the total number of eggs on a given day of the experiment, and TEB is the mean total number of eggs daily in the last 5 d before the onset of food manipulation. Thus, we assumed that if REP in a given tank under food manipulation is 1.0, the number of eggs produced should be identical to that under satiation.

Statistical analyses

Statistical analyses were performed with the software R version 3.0.2 (R Development Core Team 2013). To determine the effects of temperature, BL, and CF on VE, VL, and OGR, we used a generalized linear model incorporating a Gaussian or gamma response distribution coupled with identity or log link function to connect the mean to the linear predictors. Explanatory variables were assessed for possible collinearity visually and using variance inflation factors (VIFs) in the package DAAG (all models examined had VIF < 1.99). All model fittings were conducted using stepwise backward selections based on Akaike's information criterion (AIC). The best model of each trait was selected from the potential models with different family and link functions based on the minimum AIC. A chi-squared test was also used for analysis of deviance.

Differences in RBF, RE, and TRE among specimens collected in different temperature regimes were tested using a generalized linear model incorporating a Gaussian response distribution coupled with identity link function to connect the mean to the lin-

ear predictors. Treatments were classified into 3 categories, namely, 20°C, 24°C, and 28°C, so that the best model was selected from the 5 groupings of treatment based on the minimum AIC.

The effects of temperature on the size and C, N, and C:N, as proxies for nutritional contents, of a produced egg were analyzed using linear mixed effect (LME) models with restricted maximum likelihood estimation in the package lme4. Each trait model included temperature as a fixed effect and food type as a random effect. The food type was classified into 3 categories: New Artec, Torafugu EP, and transition from New Artec to Torafugu EP.

Differences in CF and RBF among or between treatments of food rations were tested by LME using restricted maximum likelihood estimation, with tank included as a random effect. For CF, treatments were classified into 4 categories (initial, HF, LF, and ST), so that the best model was selected from the 15 groupings of treatment based on the minimum AIC.

To examine the effects of food availability on reproductive traits during the experiment, LME with restricted maximum likelihood estimation was fitted to the data on EV and REP on the day of the experiments. A female Japanese anchovy cannot spawn every day, and batch fecundity was shown to be positively correlated with body size (Yoneda et al. 2013). It was assumed that the daily REP in each tank could vary with the total number of females spawned and

their size-dependent egg production on a given day of the experiment. Therefore, to determine changes in REP over time in each tank, the duration of the experiment under food manipulation was classified into 3 periods (early: 0–4 d, middle: 5–9 d, late: 10–14 d). In addition, we considered that the trends in REP would vary among food treatments. Thus, the LME model was run separately in each food treatment for REP. In these analyses, the full models combined period of experiment, and experiment number (Expt 1 or 2) as fixed effects and tank and day of experiment as random effects. In the EV analysis, the source of treatment was also added as a fixed effect in the full model. In all models, we started with a full model and sequentially dropped non-significant variables. Thus, the final models included only significant terms.

RESULTS

Temperature-mediated variations in reproductive traits

VE and VL were negatively correlated with temperature (Table S1 in the Supplement, Fig. 1a,b), resulting in a decrease in the increment of oocyte volumes during the period from early to late vitellogenesis in water with increasing temperature (Table S2).

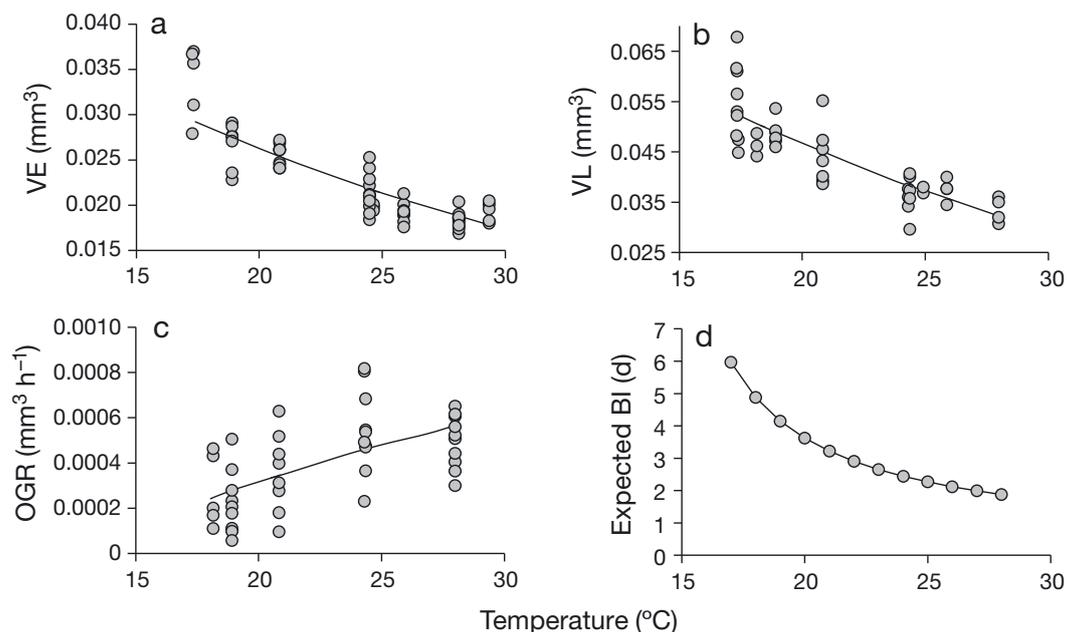


Fig. 1. Relationships of (a) oocyte volume just after spawning (VE; $n = 66$) and (b) at completion of vitellogenesis (VL; $n = 44$), (c) oocyte growth rate (OGR; $n = 44$), and (d) expected batch interval (BI) with temperature in Japanese anchovy *Engraulis japonicus*. Generalized linear models fitted to observed data are shown, except for the expected batch interval-temperature regression

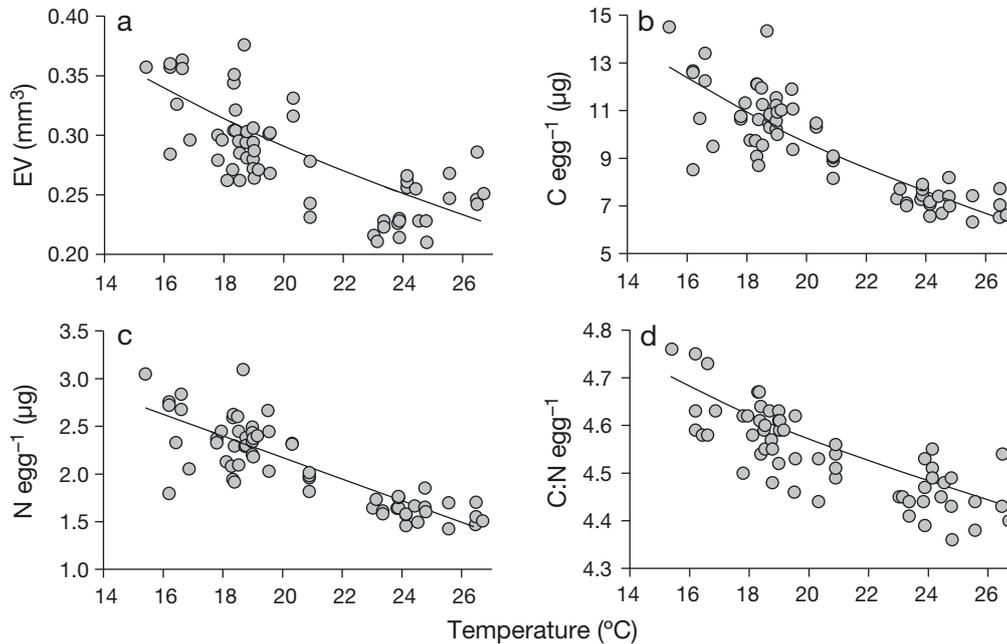


Fig. 2. Relationships of (a) egg volume (EV), the amounts of (b) carbon and (c) nitrogen, and (d) the ratio of carbon:nitrogen per egg with temperature in Japanese anchovy *Engraulis japonicus*. Linear mixed effect models fitted to the observed data are shown

Conversely, OGR increased in water with increasing temperature (Fig. 1c). As such, the duration of vitellogenesis during the period from early to late vitellogenesis decreased in water with increasing temperature: the estimated duration was 71.9 h at 20°C, while it was 26.2 h at 28°C. The estimated mean daily spawning time was 22:24 h at 20°C, while it was 20:50 h at 28°C. Thus, the BI was shortened in water with increasing temperature (Fig. 1d): estimated BI was 3.93 d at 18°C, while it was 1.96 d at 28°C. BL had a significant effect on VE, but BL and CF were not selected as significant factors in the models of VL and OGR (Table S1).

EV was negatively correlated with temperature (Table S3, Fig. 2a). The amounts of C and N of an egg were also negatively correlated with temperature (Fig. 2b,c). Similarly, the C:N ratio of an egg significantly decreased in water with increasing temperature (Fig. 2d). This was caused by a difference in the relationship between the amounts of C and N per dry egg weight and temperature: the former was negatively correlated with temperature, while the latter gradually increased with decreasing temperature (Table S3).

A generalized linear model showed no significant effect of temperature on RBF ($\chi^2 = 1.5$, $p = 0.46$; Fig. 3a). The effect of temperature on RE was significant, and the selected model showed that RE decreased in water with increasing temperature ($\chi^2 =$

29.8, $p < 0.001$; Fig. 3b). TRE was also significantly influenced by temperature; the selected model showed that TRE increased in water with increasing temperature ($\chi^2 = 49.2$, $p < 0.001$; Fig. 3c).

Effect of short-term food availability on reproductive traits

LME showed that the effect of food availability on CF in Expt 1 was significant, and the selected model showed that CF of specimens at HF was the highest of all treatments ($F_{2,182} = 23.5$, $p < 0.001$; Fig. 4a). The CF at the onset of the experiment was similar to that in LF. In Expt 2, the CF was also significantly influenced by food availability, and the selected model showed that the CF at the end of the experiment decreased as food rations were reduced ($F_{2,149} = 59.0$, $p < 0.001$). The CF at the onset of the experiment was similar to that at HF.

EV was significantly influenced by food rations and the experiment number (Table 2), but the period of the experiment did not affect EV ($F_{2,168} = 1.23$, $p = 0.29$). LME predicted that EV at LF and ST was about 4% and 7% smaller than that at HF, while EV in each treatment in Expt 1 was about 16% larger than that in the counterparts in Expt 2.

REP at HF showed higher variability during the periods of the 2 experiments, but LME showed that

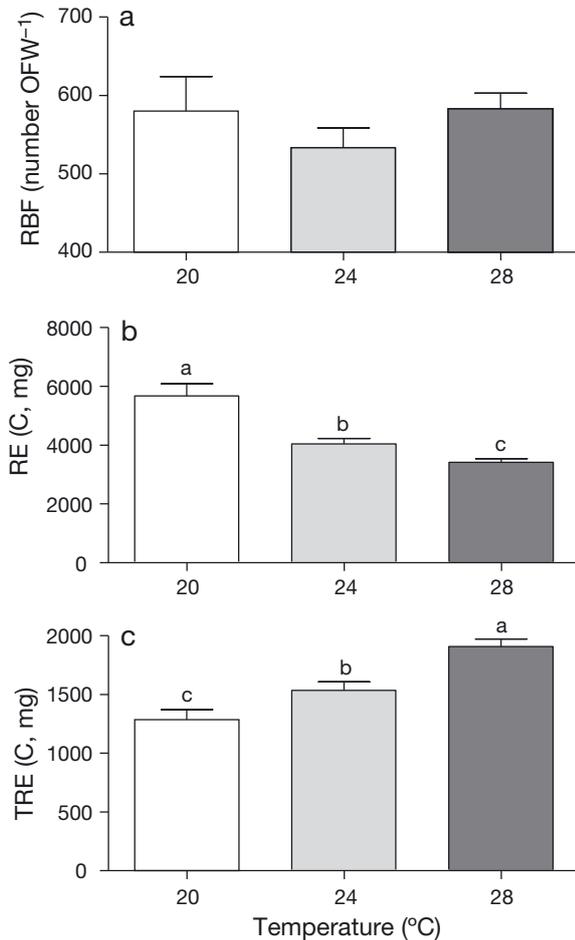


Fig. 3. (a) Relative batch fecundity (RBF; mean \pm SE), (b) carbon-based reproductive effort (RE) per spawning, and (c) total reproductive effort (TRE) at a given period of time of Japanese anchovy *Engraulis japonicus* in water at 3 different temperatures (20°C: n = 21; 24°C: n = 28; 28°C: n = 82). Different letters indicate different treatment groups in the best generalized linear model (a > b > c; p < 0.001). OFW: ovary-free weight

REP at HF remained at 0.99 throughout Expts 1 and 2 (Table S4, Fig. 5): the effects of period ($F_{2,57} = 0.28$, $p = 0.76$) and experiment number ($F_{1,58} = 0.36$, $p = 0.55$) were not significant. REP at LF decreased as the experiment progressed in Expt 1 and Expt 2. The period and experiment number had significant effects on REP at LF, resulting in lower REP in Expt 2 than in Expt 1. REP at ST drastically decreased between the early and middle periods of the experiments and thereafter remained low in the late period. The experiment number did not have a significant effect on REP at ST ($F_{1,56} = 0.92$, $p = 0.34$).

LME showed that the food ration had no effect on RBF (mean \pm SE) at the end of the experiments (Expt 1: $F_{1,26} = 3.56$, $p = 0.070$, HF: 505 ± 50 , n = 11, LF: 552 ± 32 , n = 17; Expt 2: $F_{1,26} = 3.47$, $p = 0.074$; HF: 484 ± 29 , n = 15, LF: 400 ± 41 , n = 13).

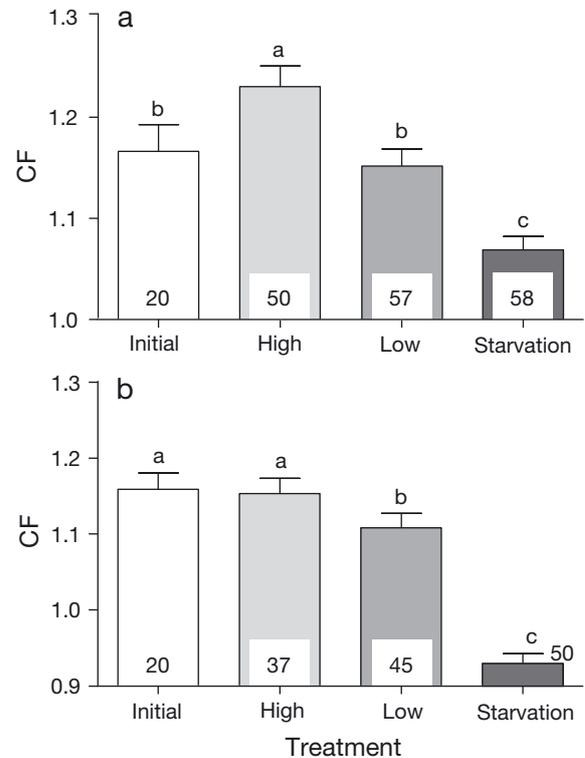


Fig. 4. Condition factor (CF; mean \pm SE) of Japanese anchovy *Engraulis japonicus* on the days at the start and end of the experiments with different food ration treatments during the 2 experiments under (a) low (Expt 1) and (b) high temperature regimes (Expt 2). Different letters indicate different treatment groups in the best linear mixed effect model (a > b > c; p < 0.001). Sample sizes are shown inside or outside bars

Table 2. Summary of linear mixed effects model estimates fitted using restricted maximum likelihood for egg volume of Japanese anchovy *Engraulis japonicus* in relation to food ration (high, low, starvation) and experiment number (Expt 1 or 2)

Model summary	Value	SE	df	F	p
Intercept	0.29	0.0047			
Food (low)	-0.010	0.0057	2, 169	5.10	0.007
Food (starvation)	-0.018	0.0057			
Experiment (Expt 2)	-0.044	0.0046	1, 169	87.8	<0.001

DISCUSSION

Our findings show how variations in prevailing temperature and food availability determine the reproductive investment of multiple-batch-spawning Japanese anchovy, which would maximize female fitness. Under ad libitum food availability, EV and BI were highly constrained by prevailing temperature.

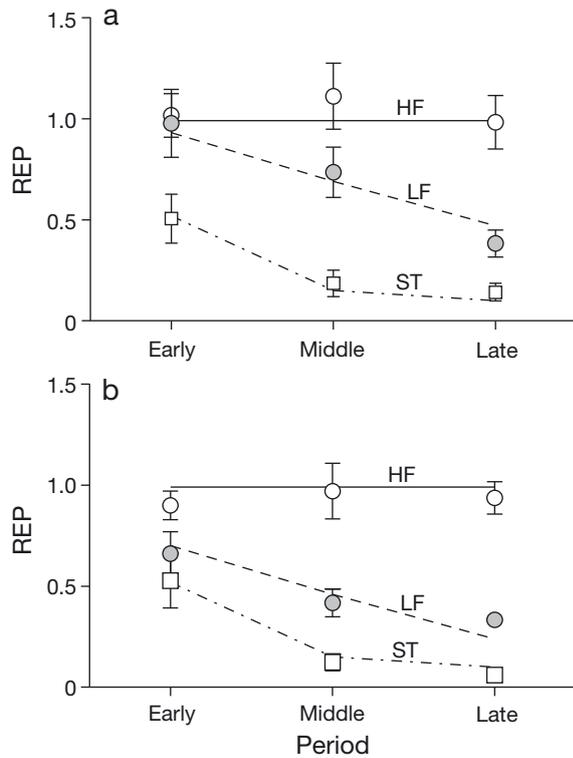


Fig. 5. Relative egg production (REP; mean \pm SE) of Japanese anchovy *Engraulis japonicus* under 3 different food treatments (high: HF, open circles; low: LF, closed circles; starvation: ST, open squares) during the 2 experiments at (a) low (Expt 1) and (b) high temperatures (Expt 2). Lines indicate expected values from the linear mixed effect models fitted to the observed data

This agrees with previous field and laboratory observations of Japanese anchovy (Tsuruta & Hirose 1989, Kawaguchi et al. 1990, Takasuka et al. 2005). Our findings also suggest temperature-dependent thresholds of size and growth of vitellogenic oocytes. As such, the effect of temperature on reproductive effort per spawning was evident even in well-fed females. There is no evidence of a trade-off between the size and number of eggs produced in this batch-spawning fish under various temperatures and nutritional conditions. Given the importance of reproductive potential for life history strategy and population dynamics, understanding which potential contributory factors have the greatest influence on reproductive allocation is essential for predicting potential population trajectories in the face of environmental change.

BI and EV are influenced by the prevailing temperature that females experience, but RBF is independent of thermal conditions under ad libitum feeding condition, the latter of which contrasts with the field observations of this species (Takasuka et al. 2005).

Such temperature-mediated variations are primarily caused by temperature-related variations in OGR and VL. These parameters are not significantly influenced by individual body conditions. Our assumption is that the variation in BI of individuals would largely be attributable to the duration of oocyte growth during vitellogenesis, as vitellogenesis appears to have been completed by 00:00 h the day before spawning (Yoneda et al. 2013). Accelerating OGR and reducing size increment from early to late vitellogenesis resulted in shorter BI at higher temperature, in addition to an earlier mean daily spawning time. The latter implies that final oocyte maturation and ovulation may also be temperature dependent (Kurita et al. 2010). Thus, these plastic responses of BI and EV produced could be reversible over a season under ad libitum feeding conditions.

As found in the variation in EV, the amounts of C and N per egg were also temperature dependent, with an egg containing higher levels of C and N in cooler water. If increased size and nutrients of an egg at lower temperature are caused only by the deceleration of physiological processes leading to reduced growth rates and extended development times of vitellogenic oocytes, the C:N ratio per egg would have remained constant across temperatures. However, such an assumption is not consistent with our findings that the C:N ratio per egg significantly decreased in water with increasing temperature. This is derived from the evidence that the C per dry egg weight dramatically decreased in water with increasing temperature, while the N per dry egg weight was positively correlated with temperature. Elemental C:N ratios of bulk tissue are usually represented as a proxy for lipid content (Logan et al. 2008), which is a source of more energy than protein and carbohydrates. Lipids are a main component of fish eggs (Kamler 2008, Fuiman & Faulk 2013), and the quantity and quality of lipids in eggs could affect growth and survival at the early stage of life (Berkeley et al. 2004). On the other hand, RBF is not significantly influenced by temperature regimes, and batch fecundity is positively correlated with somatic weight (Yoneda et al. 2013). These findings imply that females may allocate a proportional amount of energy relative to their size to the rate of egg production (batch fecundity) over a season. Consequently, RE is significantly decreased in water with an increasing temperature due to a reduction in the C content of an egg. This appears to be consistent with thermal adaptation theory (Pörtner et al. 2006), according to which a low temperature may lead to reduced costs of somatic maintenance and concomitantly a higher

proportion of resources available for egg nutrition. Therefore, females could invest more energy in reproductive output per spawning at a lower temperature than at a higher temperature.

In the food manipulation experiments, the REP in HF remained constant at 0.99 throughout the experiments involving different temperature regimes. This implies that the physiological process of converting resources to eggs would not be limited before the onset of food manipulation. As expected, our short-term food manipulations resulted in significant decreases in REP at LF and ST over the periods of the experiments. These observations may support the assumption that female Japanese anchovy could largely be dependent on income resources for egg production in successive batches over a season (Tsuruta & Hirose 1989, Kawaguchi et al. 1990). Specimens at HF showed no evidence of a decrease in CF between the initial and end stages of Expts 1 and 2. Rather, in cooler water, surplus energy may be allocated to somatic growth as the CF at HF increased during the period of Expt 1. Although we were unable to control for individual food intake, the variation in individual somatic growth trajectories must be a function of the net food intake. It is likely that well-fed females utilize surplus energy for both somatic growth and reproduction in cooler water, while in water with a higher temperature, allocation of such energy to reproduction is prioritized, possibly due to the energy costs for maintenance (Jobling 1993, Pörtner et al. 2006). Starvation resulted in significant decreases in CF, EV, and REP during the experiments, but daily spawning in tanks occurred even at the end of the experiments. Successive spawning of female Japanese anchovy in a tank was also found in a previous study in which a similar starvation experiment was conducted (Kawaguchi et al. 1990). These findings suggest that female Japanese anchovy have the potential to exhibit a mixture of capital and income breeding patterns (McBride et al. 2013), but the use of capital energy may be marginal in terms of reproductive allocation strategy.

Restricted food manipulation appears to induce thermal dependence of energy allocation to egg production, as the REP at LF differed between the periods of experiments subjected to high and low temperatures. Such thermal-dependent allocation, however, would be detrimental when income resources become unavailable, from the result of the effect on REP at ST. In the late period of the experiments, the estimated REP at LF was about 47% in Expt 1 and 24% in Expt 2 of that at HF. Interestingly, there were no significant differences in RBF between

HF and LF in Expts 1 and 2. These findings suggest that a difference in REP between HF and LF could largely be attributable to the variation in BI of individual fish. The effect of food availability on EV was significant, but the EV at LF was only ~4% smaller than that at HF. It is likely that insufficient income energy intake may lead to deceleration of OGR relative to the prevailing temperature, resulting in vitellogenesis taking longer. As such, female Japanese anchovy may maintain reproductive effort per spawning at the expense of more spawning events in a nutritionally harsh environment. Thus, although the TRE of specimens fed ad libitum increased in water with increasing temperature due to shortened BIs, this variation may be caused by a compensatory response to the level of income resources available.

Our findings of the relationships between reproductive parameters and food availability appear to be consistent with the observations of three-spined stickleback *Gasterosteus aculeatus* (Wootton & Fletcher 2009). Similar food-manipulation experiments over the spawning season suggested that the income resources could support both egg production and somatic growth. However, batch fecundity and egg characteristics (e.g. size and lipid content) are relatively resistant to variation in feeding, while spawning frequency is largely sensitive to the amount of food intake. This also agrees with findings for wild European anchovy *Engraulis encrasicolus* (Somarakis et al. 2012). The relationships between spawning parameters and body condition suggested that spawning frequency could respond more rapidly to fluctuations in food supply than batch fecundity. Conversely, in northern anchovy *E. mordax*, batch fecundity and spawning frequency remained relatively constant at different food rations and/or over the season, as egg production should largely be dependent on the level of fat energy stored before the spawning season (Hunter & Leong 1981). Our findings showed that the variation in BI of individual female Japanese anchovy can be influenced by the amount of food intake, prevailing temperature, and their interaction, which could regulate the rate of oocyte development during vitellogenesis. This potential mechanism of variation in BI could promote the bet-hedging strategy under fluctuating environmental conditions (Wright & Trippel 2009, Lowerre-Barbieri et al. 2011, McBride et al. 2013).

In conclusion, our study demonstrates that, in fish with multiple batch spawning, the interaction between temperature and income resource availability affects somatic conditions and reproductive outputs, but the size and nutrient content of eggs and BIs are

highly constrained by the prevailing temperature. In cooler water with favorable feeding conditions, females can invest more energy in reproductive output per spawning due to the production of eggs with higher nutritional content. In this situation, both females and their offspring may have a fitness advantage. This is because the surplus production also appears to be allocated to somatic growth, leading to an increase in batch fecundity, and the size and nutritional content of eggs generally reflect the likelihood of survival and growth potential of larvae (Berkeley et al. 2004, Castro et al. 2009). Conversely, shorter BIs in water with increasing temperature lead to increased TRE in a given period of time. Variation in BI, however, is also highly constrained by the amount of income resources available. Frequent spawning events in warmer water might thus be optional as prey availability in the sea is largely unpredictable (Marshall et al. 2008). These findings provide key insight into the reproductive allocation of batch-spawning species in environments with fluctuating conditions.

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