Abstract—The duration of spawning markers (e.g. signs of previous or imminent spawnings) is essential information for estimating spawning frequency of fish. In this study, the effect of temperature on the duration of spawning markers (i.e., oocytes at early migratory nucleus, late migratory nucleus, and hydrated stages, as well as new postovulatory follicles) of an indeterminate multiple-batch spawner, Japanese flounder (*Paralichthys olivaceus*), was evaluated. Cannulation was performed to remove samples of oocytes, eggs, and postovulatory follicles in individual females at 2–4 hour intervals over 27–48 hours. The duration of spawning markers was successfully evaluated in 14 trials ranging between 9.2° and 22.6°C for six females (total length 484–730 mm). The durations of spawning markers decreased exponentially with temperature and were seen to decrease by a factor of 0.16, 0.36, 0.30, and 0.31 as temperature increased by 10°C for oocytes at early migratory nucleus, late migratory nucleus, and hydrated stages, and new postovulatory follicles, respectively. Thus, temperature should be considered when estimating spawning frequency from these spawning markers, especially for those fish that do not spawn synchronously in the population.

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The effect of temperature on the duration of spawning markers—migratory-nucleus and hydrated oocytes and postovulatory follicles—in the multiple-batch spawner Japanese flounder (*Paralichthys olivaceus*)

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Production of eggs in fish populations varies spatially and temporally during a single spawning season (Scott et al., 2006; Allain et al., 2007) and interannually (Kjesbu et al., 1998). These variations can be caused by changes in temperature, food supply (Somarakis et al., 2004, 2006), fish size composition, and nutritional condition of spawners (Motos, 1996; Marteinsdottir and Begg, 2002; Scott et al., 2006). Spatial and temporal variations in egg production probably affect reproductive success (Kjesbu et al., 1996b; Wright and Gibb, 2005; Secor, 2007; Nishimura et al., 2007; Wright and Trippel, 2009). Therefore, it is necessary to understand the mechanism of spatiotemporal variation in egg production at the population level. Based on the above cited information, the variations in egg production of multiple-batch spawners can be evaluated by using accurate estimates of batch fecundity and spawning frequency in relation to age, size, and condition.

Quantification of population egg production per day is also essential for estimating population size (spawning stock biomass [SSB]) when using the daily egg production method (DEPM; Parker, 1980; Lasker, 1985). DEPM has so far been applied mainly to some clupeid fish populations and is expected to be applied to other fish species including demersal fish (Stratoudakis et al., 2006). Accurate estimation of spawning frequency is important because the largest source of error in the estimation of SSB with DEPM is believed to be associated with spawning frequency (Stratoudakis et al., 2006).

Spawning frequency (S) is expressed as the average number of spawnings per individual per day or as spawning interval (day) (in this study, the former definition is adopted). In both cases, it is estimated from the fraction (F) of mature fish that have a trait (spawning marker) related to imminent or recent spawning, e.g., oocytes at migratory nucleus (MN) or hydrated (HD) stages indicating imminent spawning, and postovulatory follicles (POFs) indicating recent spawning (Hunter and Goldberg, 1980; Hunter and Macewicz, 1985). In the case of some clupeid fish populations, it is sufficient to have a rough estimate of the duration of a spawning marker, e.g., day-0 POF, day-1 POF, etc., because these populations spawn synchronously during

a restricted period of a few hours in the early night, and sampling time is restricted to just after spawning (Hunter and Goldberg, 1980; Hunter and Macewicz, 1985; Funamoto and Aoki, 2002). However, for many other species, spawning time is not synchronized at the population level (i.e., egg release by the population is not restricted to a few hours of the day) or the sampling period for these species is broader. In this case, spawning frequency (S: 1/day) is corrected by the duration (t_i) of a spawning marker i as follows:

$$S = F \times (24 / t_i) \quad (1)$$

(Priede and Watson, 1993; Murua et al., 2003).

Many multiple-batch spawning fish have a long spawning season, and as a consequence, they spawn at a wide range of water temperatures. Therefore, the information on the duration of spawning markers in relation to ambient water temperature is critical for estimating spawning frequency accurately. So far, the duration of a stage of POF has been reported to vary depending on the ambient temperature (Fitzhugh and Hettler, 1995; Ganias et al., 2007). However, to our knowledge, temperature effects on the duration of oocytes at MN and HD stages have not been reported.

In many previous studies, the duration of spawning markers at a given temperature were evaluated by the observation of ovaries which were dissected out of periodically sampled females taken from a spawning population in the field or laboratory (Hunter and Goldberg, 1980; Hunter and Macewicz, 1985; Matsuyama et al., 1988, 2002; Shiraishi et al., 2005). This type of sampling schedule is valid when spawning occurs synchronously or over a short period (h) in the day. However, many fish species, e.g., European plaice (*Pleuronectes platessa*) and Atlantic mackerel (*Scomber scombrus*) have a relatively long spawning-time distribution (in hours per day) at the population level (Ferraro, 1980; Nichols, 1989; Walsh and Johnstone, 1992; Nichols and Warnes, 1993; Scott et al., 1993), and consequently, it is not possible to accurately estimate the duration of spawning markers by fish-group-based studies. For these fish, periodical observations of the presence of spawning markers should be taken from the same fish for a period of 24 hours. Periodic sampling of oocytes and POFs from individual captive fish by cannulation (e.g., Kjesbu et al., 1996a; Kennedy et al., 2008; Witthames et al., 2010), is a promising method for estimating the duration of spawning markers.

In this study, we used Japanese flounder (*Paralichthys olivaceus*) (also called the "bastard halibut") as a model species. The flounder is a commercially important bottom fish that inhabits coastal areas <150 m in depth. This species is a multiple-batch spawner and the most active fish spawn every day (Hirano and Yamamoto, 1992). A batch of advanced oocytes among vitellogenic oocytes (ca. 300–600 µm) enters the final oocyte maturation process and is ovulated (Kurita and Kjesbu, 2009). This species is also a typical indeterminate spawner that continues to produce vitellogenic oocytes during the spawning season (Murua and Saborido-Rey, 2003). Each individual female typically spawns over a period of three months (Hirano and Yamamoto, 1992), and the spawning season of the population lasts 4–5 months (Takeno et al., 1999). Japanese flounder experience temperatures of 7–19°C during their spawning season (Y. Kurita, unpubl. data). Spawning occurs throughout 24-h periods at the population level (Y. Kurita, unpubl. data). Thus, the duration of spawning markers during final maturation in relation to temperature is crucial information for accurately estimating spawning frequency.

The objective of this study is to evaluate the effects of temperature on the duration of spawning markers (i.e., oocytes at the MN and HD stages, and POFs) of Japanese flounder by successive sampling of ovarian tissue from individuals with a catheter over a range of ambient temperatures typically encountered during the spawning season.

Materials and methods

Experiments

To evaluate the duration of spawning markers over a range of temperatures, oocyte developmental stages relating to final maturation, and as well as POF degeneration stages (Table 1) of individual captive females were documented periodically. Two kinds of analyses were conducted: 1) an approximate evaluation of the duration for those markers; and 2) a fine-scale evaluation of the duration of hydrated oocytes.

Spawning females were held separately with two males each in cylindrical tanks with a diameter of 2.5 m and a water depth of 1 m (ca. 5 m^3 in volume) during the period covering the spawning season. A constant flow of seawater at ambient temperature was provided. Ovary samples of 1–1.5 mL were taken from each individual every 2–4 hours through 27–48 h with a thin (inner diameter; 2 mm) soft catheter (Pipelle de Cornier, Laboratoire C.C.D., France). At each catheterization, fish were weakly anesthetized with 0.08% 2-phenoxyethanol sea water for 30–120 seconds until fish did not beat their tails when they were turned over in the water. Samples were fixed in 3.6% phosphate buffered formaldehyde and subjected to the following analyses.

Samplings were conducted for 27–48 hours for a total of 14 trials (four trials for 27 hours and 10 trials for 48 hours) for six females (total length [TL] 484–730 mm) and with ambient temperature ranging from 9.2° to 22.6°C (Table 2). Out of 14 trials, the duration of all stages of oocytes during final maturation, i.e., early migratory nucleus (MN[E]), late migratory nucleus (MN[L]), and HD stages, and new postovulatory follicles (POF[new]) (Table 1) were monitored for five trials (three females; TL 605–710 mm); MN(E), MN(L), and POF(new) for one trial (one female; TL 605 mm); and MN(L) and HD for another trial (one female; TL 730 mm). Moreover, the duration and growth rate of only

Table 1

Criteria for each developmental stage of oocyte, ovulated egg, and postovulatory follicle of Japanese flounder (*Paralichthys olivaceus*).

		Description	
Developmental stage	Abbreviation	Histology	Whole mount appearance with transmitted light
Yolk granule	YG	Yolk granules present. Many small oil droplets are distributed around the nucleus which is located in the center (Fig. 1A).	Oocytes are slightly dark and oil droplets, which are located around the nucleus, look like a dark shadow (Fig. 1B).
Early migratory nucleus	MN(E)	Yolk granules are larger. Oil droplets fuse and are distributed unevenly to one side (within 180° from the center of nucleus) beside the nucleus (Fig. 1C).	Uneven distribution of oil droplets can be recognized as dark shadow. Difficult to distinguish from YG (Fig. 1D).
Late migratory nucleus	MN(L)	Oil droplets fuse into 1–3 big droplets (Fig. 1E).	Yellowish oil droplets can be easily recognized (Fig. 1F) and usually distinguishable from MN(E) and YG.
Hydrated	HD	In the earlier phase, yolk granules fuse progressively and start to become irregularly shaped (Fig. 1G). In the later phase, all yolk glanules fuse into a uniform yolk mass that occupies the inside of the oocyte (Fig. 1I). Oocyte is still surrounded by the follicle layer.	In the earlier phase, the whole mount appearance turns opaque and dark (Fig. 1H). In the later phase the whole mount appearance turns translucent and a big yellowish oil droplet is prominent (Fig. 1J). Oocytes at this stage are easily distinguished from other stages of oocytes.
New ovulated egg	OV	A uniform yolk mass occupies the inside of the egg. Egg is free from follicle layer. Egg coexists with POF(new).	Appearance is similar to the oocyte at the late HD stage.
New postovulatory follicle	POF(new)	Granulosa cells are clearly recognized. Cell membrane and nucleus of granulosa cells are intact (Fig. 1K).	

HD-stage oocytes were monitored for seven other trials (four females; 484–710 mm TL). The former seven trials where the duration was monitored for more than two spawning markers, conducted between 9.2° and 22.6°C, were used for analyses of the approximate evaluation of the duration of spawning markers and the size range of hydrated oocytes, the latter of which is the range between the maximum and the minimum diameters of hydrated oocytes through the final maturation process at each temperature of the experiment). In total, 20 events of hydration from 13 trials conducted between 9.2° and 19.7°C were used for analyses of growth rate and the duration of HD-stage oocytes.

Histological and whole mount examination for evaluation of the duration of the spawning marker

Approximate evaluation of the duration for spawning markers was conducted as follows. A part of each cannulated sample was dehydrated and embedded in resin (Historesin), sectioned at 4 µm thickness, and stained with 2% toluidine blue and 1% borax. The occurrence of oocytes at the yolk granule (YG; Table 1), MN(E), MN(L), and HD stages, and POF(new) were recorded by a combination of histological examination under a light microscope and whole mount examination under a binocular microscope (Fig. 1). The start time of the duration of each stage was estimated as the mid-point of the two consecutive sampling times when the developmental stage was not observed and then observed. Similarly, the end time was the mid-point of the two consecutive sampling times when the developmental stage was observed and not observed. The duration of oocytes at the MN(E) and MN(L) stages, and POF(new) were estimated at each trial, and the relationships with temperature were analyzed as the exponential formulae.

Measurement of oocyte diameter for evaluation of duration of the HD stage

The duration of HD stage was estimated more precisely as the range of hydrated oocyte diameter divided by the

Table 2

Experimental data obtained from ovarian tissue from Japanese flounder (*Paralichthys olivaceus*). Ovarian tissue was examined every two to four hours over a 27- or 48-hour sampling period. MN(E)=oocyte at early migratory nucleus stage; MN(L)=at late migratory nucleus stage; HD=at hydrated stage; POF(new)=new postovulatory follicles.

Fish number	Total length (mm)	Experiment period	Duration of experiment (hour)	Temperature (°C)	Spawning markers used for evaluating marker duration or estimating hydrated oocyte growth rate[1]			
					MN(E)	MN(L)	HD[2]	POF(new)
1	605	7–9 June 2004	48	14.1	Y	Y	Y(2)	Y
1		12–14 July 2004	48	16.7	Y	Y	Y(1)	Y
1		9–11 Aug 2004	48	22.6	Y	Y		Y
2	710	30 May–1 June 2005	48	11.3(11.9)[3]	Y	Y	Y(2)	Y
2		30–31 June 2005	27	13.7			Y(2)	
2		20–22 July 2005	48	16.7			Y(2)	
3	707	30 May–1 June 2005	48	11.2(11.9)[3]			Y(2)	
3		30–31 June 2005	27	14.1			Y(2)	
3		20–22 July 2005	48	16.9	Y	Y	Y(2)	Y
3		11–13 Aug 2005	48	19.7	Y	Y	Y(1)	Y
4	484	12–14 July 2004	48	16.8			Y(1)	
5	545	30 May–1 June 2005	48	11.2			Y(1)	
5		30–31 June 2005	27	13.7			Y(1)	
6	730	23–24 May 2006	27	9.2		Y	Y(1)	
				Total	6	7	20	6

[1] "Y" indicates that data were obtained.

[2] Figures in parentheses show the number of hydration events used for calculation of the growth rate of hydrated oocytes.

[3] Figures in parentheses show the temperature at the second hydration event.

growth rate of hydrated oocytes. Changes in those two variables were evaluated in relation to temperature as follows. For the measurements of oocyte diameter, the width of follicle layer was not included.

First, the growth rate of hydrated oocytes was examined. For each cannulated sample, the diameters of 30 hydrated oocytes were measured manually in a whole mount (dispersed sample observed under a binocular microscope) with image-analysis software (Image Pro PLUS, Media Cybernetics, Inc., Bethesda, MD). Growth rate of hydrated oocytes (μm/h) was calculated as the slope of the regression line between the average hydrated oocyte diameter and sampling time. The relationship between the growth rate of hydrated oocytes and temperature was established as the exponential formula.

Next, to estimate the size range of diameters of hydrated oocytes at each temperature unit of the experiment, ovulated egg diameter and the maximum MN(L)-stage oocyte diameter through 48 hours of sampling were measured as the substitution for the maximum and the minimum hydrated oocyte diameters, respectively.

The diameter of 30 newly ovulated eggs (Table 1) that remained in the ovarian cavity from each cannulated sample were measured manually with image-analysis software and averaged. Ovulated eggs were distinguished from large hydrated oocytes by histological examination; i.e., ovulated eggs did not have follicle layers around them and coexisted with new postovulatory follicles. Diameters of ovulated eggs were measured for the two consecutive samples after the samples in which the largest hydrated oocytes in each hydration process were observed.

The diameters of an additional 200–300 fixed, whole-mount oocytes at the YG, MN(E), and MN(L) stages from each cannulated sample were also measured with image analysis software. Average diameters of oocytes at the MN(L) stage were calculated for each sample. When the MN(L) stage oocytes were distinguishable in whole-mount samples (Table 1), the diameters of these oocytes were measured and averaged. However, in some cases, the MN(L)-stage oocytes were difficult to distinguish from the MN(E)-stage oocytes in the whole-mount samples by diameter and appearance. In those cases, the proportion of the number of MN(L)-stage oocytes to the number of the developing oocytes (oocytes at the YG, MN(E), MN(L), and HD stages) was set as the same proportion of the number of HD-stage oocytes to the developing oocytes (Fig. 2). For the example in Figure 2, the proportion of MN(L)-stage oocytes to the developing oocytes was

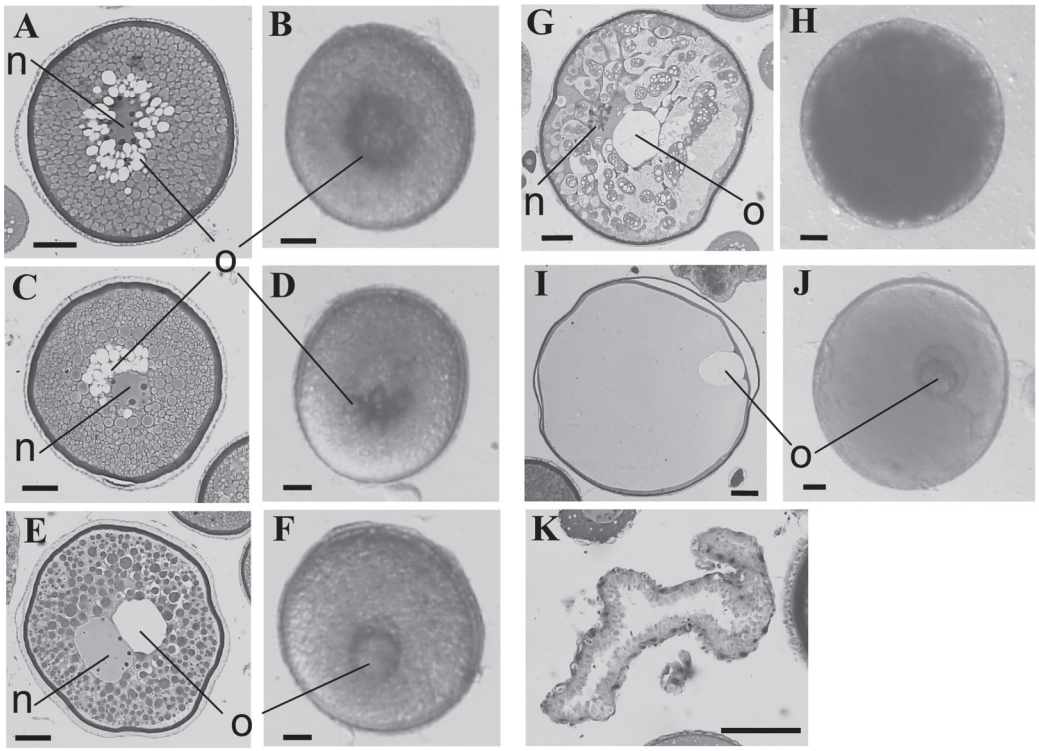

Figure 1

Histological and whole mount images of oocytes and postovulatory follicles (POFs) of Japanese flounder (*Paralichthys olivaceus*). (**A**, **B**) Oocyte at yolk granule stage; diameter ca. 500 µm. (**C**, **D**) Early migratory nucleus stage; diameter ca. 600 µm. (**E**, **F**) Late migratory nucleus stage; diameter ca. 650 µm. (**G**, **H**) Early hydrated stage; diameter ca. 720 µm. (**I**, **J**) Late hydrated stage; diameter ca. 900 µm. (**K**) New POF. Scale bars=100 µm. See Table 1 for detailed definitions. o=oil droplet; n=nucleus.

set at 4.5% which was the same proportion as that for the HD-stage oocytes.

The lower size limit of hydrated oocytes at a given temperature was considered equal to the maximum average size of MN(L)-stage oocytes among the successive 48-h samplings (Table 2) at that temperature because the growth rate of MN(L)-stage oocytes was very slow and the maximum average size of MN(L)-stage oocytes can be considered as the smallest diameter for the hydrated oocytes (see Fig. 3). The average egg diameter was considered as the maximum diameter for the hydrated oocytes. The lower and upper limits of hydrated oocyte diameters were obtained from six (9.2–19.7°C) and seven (9.2–22.6°C) time series samples, respectively. Changes in those diameters were analyzed in relation to temperature.

Average diameters of oocytes at the MN(E) stage and the leading cohort of oocytes at the yolk granule stage (YG[LC]) were also calculated for each sample. The proportions of the number of oocytes at the MN(E) and the YG(LC) stages to the developing oocytes were determined in the same manner as that for MN(L) stage (Fig. 2). The leading cohort of YG-stage oocytes is usually defined as the largest 10% of YG-stage oocytes (Kjesbu, 1994) to specify the maturity phase. In this study, however, the proportion of YG(LC)-stage oocytes to the developing oocytes was set at the same proportion of HD-stage oocytes (e.g., 4.5 % in Fig. 2) to correspond to the expected batch of oocytes.

Body size and individual effects on the growth rate of hydrated oocytes

To evaluate size effect on the growth rate of hydrated oocytes, multiple regression analysis was conducted for 20 data sets of log-transformed growth rates of hydrated oocytes (dependent variable), temperature, and total length (independent variables). In addition, to evaluate the individual effect on the growth rate of hydrated oocytes, the relationship between the log-transformed growth rate of hydrated oocytes and temperature (T) was compared for two females (fish no. 2; TL 710 mm, $11.3 \leq T \leq 16.7$°C, $n=6$: fish no. 3; TL 707

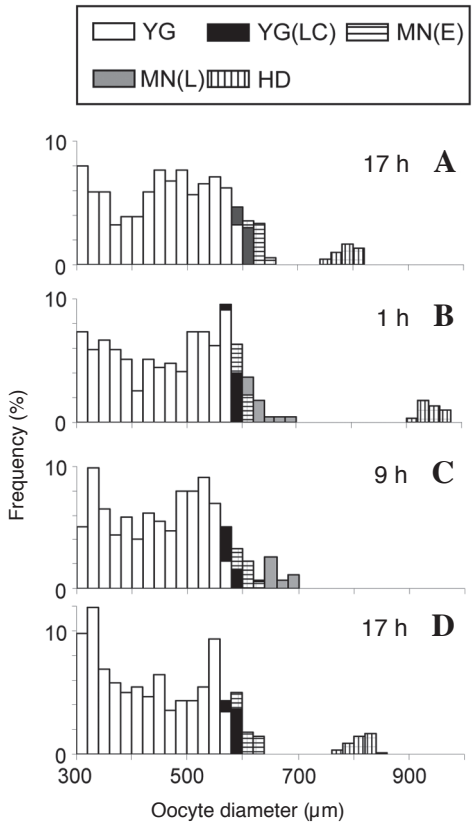

Figure 2

Temporal changes in the frequency distribution of oocyte diameter at different developmental stages of Japanese flounder (*Paralichthys olivaceus*). Data are from successive samples of oocytes from fish no. 2, 30 May-1 June 2005 (see Table 2 and Fig. 3**A**). (**A**) on 30 May, and (**B**–**D**) on 31 May. HD=oocyte at hydrated stage; MN(L)=at late migratory nucleus stage; MN(E)=at early migratory nucleus stage; YG(LC)=the leading cohort of oocytes at yolk granule stage; and YG=yolk granule stage.

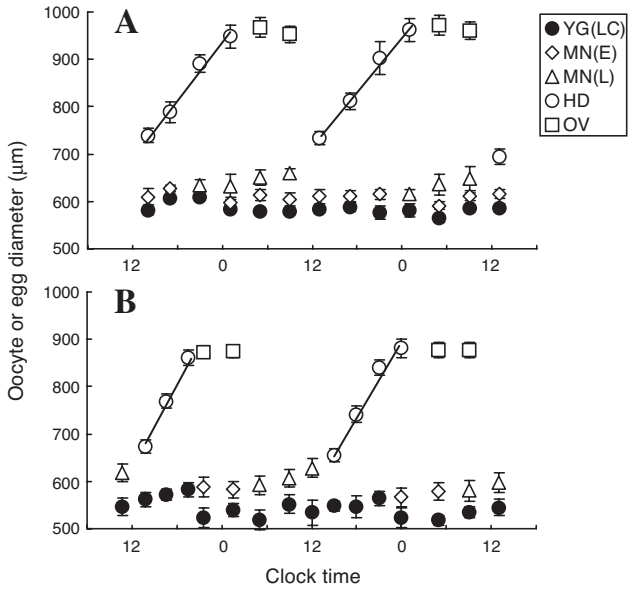

Figure 3

Diurnal changes in oocyte diameters at each developmental stage for individual Japanese flounder (*Paralichthys olivaceus*). (**A**) Experiment for fish no. 2, conducted between 30 May and 1 June in 2005 at 11.3°C. (**B**) Experiment for fish no. 3, conducted between 20 and 22 July in 2005 at 16.9°C. Bars indicate standard deviation. The slopes of regression lines for HD-stage oocytes represent the growth rate of hydrated oocytes. OV=ovulated egg; HD=oocyte at hydrated stage; MN(L)=at late migratory nucleus stage; MN(E)=at early migratory nucleus stage; and YG(LC)=the leading cohort of oocytes at the yolk granule stage.

mm, 11.2≤T≤16.9°C, n=6: Table 2) by ANCOVA. We could not examine the effect of other females because the overlap of temperature was narrow.

Results

Experiments

The growth rates of oocytes at MN(L), MN(E), and YG(LC) stages were slow, whereas the growth rate of HD-stage oocytes was rapid (Fig. 3). At the lower temperature, HD- and MN-stage oocytes, or MN(L)- and MN(E)-stage oocytes coexisted, indicating that the two batches of oocytes entered the final maturation process at the same time (Figs. 2 and 3).

In five out of ten 48-h experiments, final maturation processes were observed twice at about 24-h interval (Figs. 2 and 3). In the other five 48-h experiments, the final maturation process occurred only once on the first day of each experiment. Once a batch of oocytes entered the final maturation process, these oocytes completed final maturation to be ovulated.

Although females spawned naturally in the tank for their spawning periods of 2–3 months, they could not spawn naturally during the 48-h experiments. They could only ovulate oocytes into their ovarian cavity.

Duration of each spawning marker

The duration of each spawning marker changed considerably with temperature. The durations of oocytes at the MN(E) and MN(L) stages, and POF(new) decreased exponentially from 24 to 3 h, 16 to 4 h, and 16 to 4 h, respectively, for the established temperature ranges (9.2° or 11.3° to 22.6°C; Fig. 4). Duration of these markers decreased by a factor of 0.16, 0.36, 0.31, respectively, as temperature increased by 10°C (Table 3).

The growth rate of hydrated oocyte diameter (G_{HD}, in µm/h) increased exponentially as temperature (T) increased (Fig. 5) as follows:

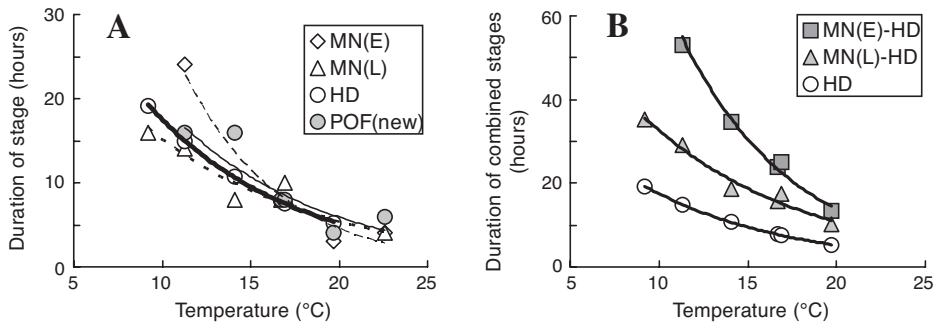

Figure 4

(**A**) Duration of oocytes at each developmental stage and new postovulatory follicles and (**B**) combined developmental stages in relation to temperature for Japanese flounder (*Paralichthys olivaceus*). MN(E)=oocyte at early migratory nucleus stage; MN(L)=at late migratory nucleus stage; HD=at hydrated stage; POF(new)=new postovulatory follicles; MN(E)-HD=combined stages of MN(E), MN(L), and HD; MN(L)-HD=combined stages of MN(L) and HD. Statistics for regression lines are shown in Table 3.

$$G_{HD} = 8.66\ e^{0.0799T},$$
$$(9.2 \leq T \leq 19.6°C,\ n=20,\ r^2=0.937,\ P<0.001). \quad (2)$$

Thus, growth rate increased by a factor of 2.2 as temperature increased by 10°C. Body size and individual effects on the relationship between the growth rate of hydrated oocytes and temperature were not observed (body size effect; multiple regression analysis, $P=0.12$: individual effect; ANCOVA, slope, $P=0.29$; intercept, $P=0.75$).

Ovulated egg diameters (*ED*, µm), as a substitution for the maximum hydrated oocyte diameter, decreased from 980 to 830 µm as temperature increased (Fig. 6) as follows:

$$ED = -11.8T + 1099,$$
$$(9.2 \leq T \leq 22.6°C,\ n=7,\ r^2=0.975,\ P<0.001). \quad (3)$$

The largest diameter of oocytes at the MN(L) stage, as a substitution for the minimum hydrated oocyte diameter, did not decrease significantly with temperature ($9.2 \leq T \leq 19.6$°C, $n=6$) and averaged 645 µm. Thus, the ranges of hydrated oocyte diameter also decreased linearly from 345 to 220 µm as temperature increased from 9.2° to 19.7°C (Fig. 6). As a result, the duration of the HD stage, i.e., the range of hydrated oocyte diameter divided by the growth rate of hydrated oocytes, also decreased exponentially from 19.1 to 5.3 h as temperature increased from 9.2° to 19.7°C (Fig. 4, Table 3). Duration of this stage decreased by a factor of 0.30 as temperature increased by 10°C.

The duration of combined spawning markers, i.e., MN(L) + HD stages and MN(E) + MN(L) + HD stages, also decreased exponentially from 35.1 to 10.3 h (9.2–19.7°C) and 53.0 to 13.3 h (11.3–19.7°C), respectively (Fig. 4, Table 3).

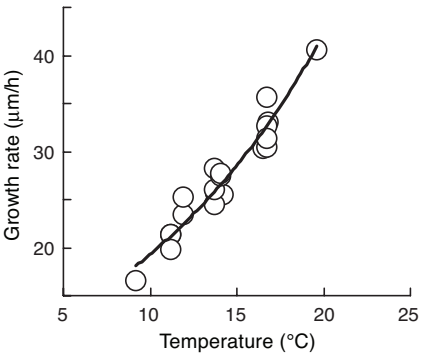

Figure 5

Relationship between growth rate of oocyte diameter during hydration and temperature for Japanese flounder (*Paralichthys olivaceus*). Each data point represents one hydration event. See Equation 2 in the main text.

Discussion

In this study, we clearly show that duration of spawning markers, i.e., oocytes at the MN(E), MN(L), and HD stages, and the POF(new) stage, largely varied because of water temperature. The duration of a marker affects estimates of spawning frequency according to Equation 1 and can have large effects if fixed spawning markers are used for a range of ambient temperatures. The duration of spawning markers decreased exponentially as temperature increased. Thus, estimates of spawning frequency per day in colder and warmer waters tend to be overestimated and underestimated, respectively, if the effect of temperature on marker duration is not

considered and if a constant estimate of duration is used. In the case of Japanese flounder, the duration of the HD stage decreased from 19.1 to 5.3 h between 9.2° and 19.7°C. If an HD-stage oocyte is used as a spawning marker and 2 out of 10 sampled females have HD-stage oocytes in their ovaries, the estimated spawning frequency by Equation 1 will be very different, i.e., 0.25 at 9.2°C and 0.91 at 19.7°C, leading to answers that differ by a factor of 3.6 (=[0.2×24/5.3]/[0.2×24/19.1]).

Each spawning marker has advantages and disadvantages for estimating spawning frequency. The merit of using POFs as spawning markers is that females with day-1 or day-2 old POFs are usually found away from spawning aggregations and estimates of spawning frequency are therefore less biased (Hunter and Goldberg, 1980; Picquelle and Stauffer, 1985; Ganias et al., 2003). However, aging or staging is difficult because the process of degeneration is continuous (Ganias et al., 2003). On the other hand, the merit of using oocytes at the MN and HD stages as spawning markers is that aging or staging is easy. However, in some cases estimates of spawning frequency with the MN- or HD-stage oocytes provide biased estimates because of the nonuniformity of their distribution, i.e., during spawning aggregations. Therefore, in cases where fish populations spawn within a restricted period of a few hours, as occurs with some clupeid fish populations (Stratoudakis et al., 2006) and sampling is also conducted within a restricted time period, the POF is an appropriate spawning marker because staging is easier as the age of POF appears intermittently (Hunter and Goldberg, 1980; Hunter and Macewicz, 1985). But when fish spawn over a prolonged period (over many hours in a day) at the population level, or when sampling time is not restricted to a short period, oocyte at the MN or HD stage is appropriate spawning marker because aging or staging is much easier than POFs, and the use of Equation 1 is recommended. Even in species that form spawning aggregations around spawning events, a MN-stage oocyte may be a useful spawning marker because oocytes at the MN stage appear several hours before spawning and the duration of the MN stage is relatively long. In any case, precise evaluation of the duration of a marker and its changes due to ambient temperature is essential, especially for species that spawn over long time periods within a day.

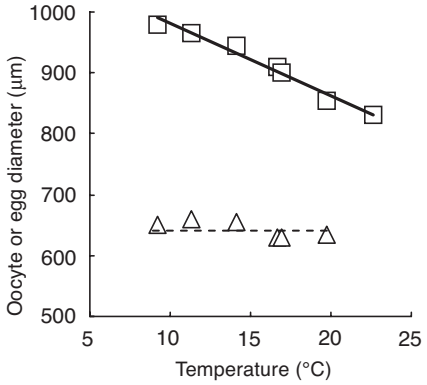

Figure 6

Changes in the range (from the minimum to the maximum) of sizes of oocytes at the hydrated stage (HD) as a function of temperature (T) for Japanese flounder (*Paralichthys olivaceus*). The minimum and maximum sizes of oocytes at the HD stage were replaced with the maximum size of oocytes at the late migratory nucleus stage (△), and with the average diameter of ovulated eggs (□), respectively, in a 48-h experiment at each temperature. The broken line shows the average diameter of the maximum sizes of oocytes at the late migratory nucleus stage (645 µm) and the solid line shows the regression line for the diameter of ovulated eggs. See Equation 3 in the main text.

Table 3

Parameters of equations used to determine stage duration (D) at temperature (T); $D=a\times e^{-bT)}$ for Japanese flounder (*Paralichthys olivaceus*) with statistics (n=number of data points; r^2=coefficient of determination; P=probability of error in the regressions). The reduction rate of D for an increase in temperature of 10°C ($D_{(T+10)}/D_T$) is also shown. MN(E)=at early migratory nucleus stage; MN(L)=at late migratory nucleus stage; HD=at hydrated stage; POF(new)=new postovulatory follicle.

Stages	a	b	$D_{(T+10)}/D_T$	n	r^2	P
MN(E)	186	0.186	0.16	6	0.88	0.006
MN(L)	42.4	0.103	0.36	7	0.90	0.001
HD(calculated)[1]	59.4	0.122	0.30	6	1.00	<0.001
POF(new)	62.9	0.118	0.31	6	0.74	0.028
MN(L) + HD	98.4	0.111	0.33	6	0.96	<0.001
MN(E) + MN(L) + HD	332	0.159	0.20	5	0.98	0.001

[1] Stage duration at each temperature was calculated with the size range and the growth rate of HD-stage oocytes (Eq. 2).

To our knowledge, this is the first report to clearly illustrate the duration and growth rate of oocytes during final maturation, on an individual-fish basis, in relation to ambient water temperature. Final maturation, in this study, is considered to have progressed normally for the following three reasons, although successive sampling in general would produce strong stress on animals. First, past *in vitro* incubation experiments of Japanese flounder at 15°C have shown that the duration of the HD stage is 12 hours and the duration of the final maturation process is about 33 hours (Matsubara et al., 1995). These results are comparable to ours, namely that the estimated duration of the HD stage is 10 hours and the duration of final maturation (MN[E], MN[L], and HD stages) is 30 hours at 15°C. Second, other studies also have reported the negative relationship between the duration of final maturation (from injection of pituitary extracts or gonadotropin-releasing hormone [GnRH] to ovulation) and ambient water temperature (common carp [*Cyprinus carpio*], Drori et al., 1994; streaked prochilod [*Prochilodus platensis*], Fortuny et al., 1988) as seen in this study. Third, hydration is driven by the osmotic gradient caused by protein hydrolysis of yolk and ion accumulation through many enzyme catalyses (Cerdà et al., 2007). The rate of enzyme catalysis increases as temperature increases, within a range of appropriate temperatures. Therefore, it is reasonable that the growth rate of hydrated oocytes increases, or in other words, duration of this stage decreases as ambient water temperature increases.

In some trials, females did not produce a new batch of oocytes that entered the final maturation process during the 48-h experiment period, probably because of the stress experienced during the sampling procedure. However, once a batch of oocytes entered the MN stage, those oocytes completed final maturation and proceeded to ovulation, even when under the stressful conditions of the experiments. This finding indicates that GnRH or gonadotropin secretion, which induce a batch of oocytes to proceed to final maturation, is likely susceptible to stress, but the process of the final maturation of oocytes is less susceptible, at least for Japanese flounder. In addition, fish could ovulate oocytes but could not spawn them during the 48-h experiment period. Spawning behaviour or the endocrine control of spawning, or both, are also likely susceptible to stress.

Successive sampling of oocytes from individual females with a catheter is a useful method for clarifying the diurnal rhythm of the final maturation of fish species, especially those with a long spawning time distribution within a day at the population level. However, careful consideration should be paid for reducing stress to fish during the sampling procedure. Anesthetizing and handling seem to be the main sources of stress. In this study, we tried to reduce stress by weakly anesthetizing fish for a short duration. We also did not use a scoop net to pick up fish because the fish would move around in the tank to escape the net and struggle in the net, both of which would cause undue stress and physical injury to the fish, e.g., injuries to their body surface and internal bleeding. Instead, we lowered the water level of the tank, gently picked up the fish, placing both hands under its body, put it into a crate made of styrene foam, which floated on the surface of water, carried the crate with the fish and placed the fish in a bath filled with 0.08% 2-phenoxyethanol sea water without touching the fish. During this procedure, the fish did not struggle much and seemed to be less stressed. We also experimented with taking ovary samples by cannulation without anaesthesia; i.e., we picked up the fish with both hands placed under its body, put it on a board floating on the surface of water, and then took samples of oocytes. These operations took 10–20 seconds and females stayed still on the board. The latter method seemed to be a better procedure to reduce stress.

Spawning frequency is, in general, estimated by using the fraction of fish with a spawning marker and the duration of the marker as seen in Equation 1 (Priede and Watson, 1993; Murua et al., 2003). Duration of oocytes at the MN and HD stages, in addition to POFs (Fitzhugh and Hettler, 1995; Ganias et al., 2007), changes as temperature changes. Thus, accurate estimates of the duration of a marker in relation to temperature are essential for estimating spawning frequency accurately, especially for those fish species that have long spawning-time distribution within a day at the population level and experience a wide range of ambient water temperatures during their long spawning season.

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