Quantifying fecundity in *Macoma balthica* using an enzyme-linked immunosorbent assay (ELISA)

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ABSTRACT: Monoclonal antibodies specific to a heat shock protein 70-like protein expressed in the eggs of *Macoma balthica* (mb-HSP70) were employed to develop an enzyme-linked immunosorbent assay (ELISA) to quantify fecundity in females. The assay was specific to egg tissue, with no demonstrable reactivity with juvenile or male tissues. The concentration of mb-HSP70 increased as the female gonads matured, necessitating the determination of a calibration curve for future experiments. The number of eggs in females was positively correlated with body mass index (BMI), and clams with a BMI <1.4 did not produce eggs. The estimated number of eggs per clam during the fall spawning period was similar to that observed in eastern North Atlantic populations in clams of similar size; clams with shell lengths from 16 to 18 mm had from 7000 to 60 000 eggs apiece. Larger clams had up to 450 000 eggs. This assay effectively quantifies eggs at any stage of gonadal development in which eggs can be distinguished microscopically and is easier and cheaper to perform than other techniques of comparable precision.

KEY WORDS: *Macoma balthica* · Fecundity · Egg · ELISA · Monoclonal antibodies · Heat shock protein

INTRODUCTION

The tellenid clam *Macoma balthica* is a small, thin-shelled clam common in estuaries in both the eastern and western North Atlantic. In the Chesapeake Bay, it is the biomass-dominant organism in mesohaline, muddy habitats, comprising up to 85% of the biomass (Holland et al. 1977). In the Chesapeake, *M. balthica* is an important link in the food web, as a variety of piscine and crustacean predators prey upon it; *M. balthica* comprises approximately 50% of the blue crab *Callinectes sapidus* diet alone (Hines et al. 1990). In eastern North Atlantic estuaries, it is an important food supply for migratory shorebirds (Stillman et al. 2005). It is considered an indicator of ecosystem health, as it is relatively sensitive to stressors such as eutrophication and hypoxia (Seitz et al. 2003, Powers et al. 2005).

The reproductive cycle of *Macoma balthica* has been well studied, although its seasonality varies among populations. In the Chesapeake, *M. balthica* spawn in both the fall and spring (Shaw 1965), whereas in Massachusetts and in the eastern North Atlantic they only spawn in the early spring (Gilbert 1978, Honkoop & van der Meer 1998). *M. balthica* invests heavily in eggs, producing large, energy-rich eggs with high lipid content that can account for >30% of their mass (Honkoop et al. 1999). Reproductive output varies with water temperature (Honkoop & van der Meer 1998), food quality (Hendriks et al. 2003), and the body mass index (BMI) of the clam (Beukema et al. 2001).

Estimating the reproductive success of an individual is difficult, especially for broadcast-spawning marine invertebrates, such as *Macoma balthica*, because of the many factors that affect fertilization success and

the survival and successful settling of larvae. The number of eggs successfully fertilized is dependent, not only on the number spawned, but also on the timing of spawning (i.e. synchronized spawning), the population density, environmental conditions, such as water flow, turbulence, and quality, and other factors (Levitan 1995). Once fertilized, the embryos and later larvae must face mortality from such factors as physiological stress, starvation, and predators before they can successfully settle and recruit (Morgan 1995). Because of the difficulty in measuring these processes, the reproductive potential of an individual is often estimated as the fecundity or number of gametes present in the individual prior to spawning (e.g. Kang et al. 2003b), though this may be an overestimate, as some eggs may be reabsorbed by the female prior to spawning (e.g. Al-Mohanna et al. 2003).

Measuring fecundity in *Macoma balthica* and in other bivalves is difficult, and various methods have been used. Visual examination and ranking of gonadal fullness is an easy, but highly qualitative measurement (e.g. Gilbert 1978, De Goeij & Honkoop 2003). Tissue sectioning and manual counting of eggs under a microscope is more quantitative, but labor intensive (e.g. Kang et al. 2003a). A common technique used with *M. balthica* is induction of spawning via temperature shock, followed by collection and counting of eggs, which is quantitative, but also labor intensive (e.g. Honkoop et al. 1999, Beukema et al. 2001).

An easier technique, enzyme-linked immunosorbent assay (ELISA), has been developed to quantify eggs in a variety of bivalves, including *Crassostrea virginica* (Choi et al. 1993), *Crassostrea gigas* (Kang et al. 2003b), *Saxidomus purpuratus* (Park et al. 2003) and *Ruditapes philippinarum* (Park & Choi 2004). ELISAs allow for rapid, simultaneous, quantitative assessments of a large number of samples. In the present study, we developed a sandwich ELISA, using monoclonal antibodies to quantify the number of eggs in *Macoma balthica*.

MATERIALS AND METHODS

Production of antibodies and purification of egg protein standard. Monoclonal antibodies to egg-specific proteins in *Macoma balthica* were produced using subtractive immunization (see Bromage et al. 2008, in this issue, for detailed methods). Briefly, mice were injected with a crude protein extract of juvenile (i.e. non-reproductive) *M. balthica* homogenized in phosphate-buffered saline (PBS; 8.4 mM Na_2HPO_4, 1.6 mM NaH_2PO_4, 150 mM NaCl, pH 7.2) and centrifuged at $15\,000 \times g$. Subsequent cyclophosphamide injections killed all proliferating B-cells. The mice

were then injected with a crude protein extract of *M. balthica* eggs homogenized in PBS and centrifuged at $15\,000 \times g$. This technique ensured that any B-cells that proliferated produced antibodies specific to targets unique to the egg extracts (Matthew & Sandrock 1987). B-cells were isolated and fused with myeloma cells, and the resulting Hybridomas were screened with ELISA against both juvenile and egg extracts to identify positive clones and verify specificity. For this study, we used monoclonal antibody 7A4. We determined, via Western blotting and protein sequencing, that it was specific to a 96 kDa *M. balthica* egg-specific HSP70-like protein (mb-HSP70; Bromage et al. 2008). It was stored at 1 mg antibody ml^{-1} in 50% glycerol at –20°C.

Ripe female *Macoma balthica* were collected from the York River, Chesapeake Bay on 23 October 2003 and stored at –20°C before use. In this collection, as in all others, we collected more clams than we thought we would need (typically 50 to 150 clams $collection^{-1}$), and we sexed them in the laboratory when required. An egg protein standard was made by incising and squeezing the gonads to collect eggs. The eggs from 3 females were pooled, homogenized in PBS, and centrifuged at $15\,000 \times g$ for 1 h at 4°C. The supernatant was collected and the protein concentration was determined with a bicinchoninic acid assay (BCA; Sigma) using bovine serum albumin as the standard. The egg protein standard was stored at 1.25 mg protein ml^{-1} in 1 ml aliquots at –20°C. In the present paper, concentrations of mb-HSP70 will be expressed in 'units', where 1 unit of mb-HSP70 is equal to the concentration of mb-HSP70 in 1 ml of 1.0 mg ml^{-1} egg protein standard. When the first standard ran low, a second was made using 3 ripe females collected on 30 October 2005. The same procedure was followed as above, and the concentration of mb-HSP70 in the new standard was determined by ELISA (see the following subsection).

ELISA. The following ELISA protocol we developed and used to analyze all samples. ELISAs were run in 96-well polystyrene micro-plates, and all incubations were executed at 37°C. The primary antibody, 100 µl of 7A4 at 2 µg ml^{-1} in citrate buffer (10 mM sodium citrate, pH 4.0), was added to each well and incubated for 1 h. The wells were then blocked with 240 µl of Tris-buffered saline with 0.1% Tween-20 (TBST; 50 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 8.0) with 1% bovine serum albumin, and incubated for 1 h. Subsequently, 100 µl of each undiluted sample together with 1:5 serial dilutions in TBST were analyzed in duplicate. Serial dilutions of the egg standard were also run on every plate. After blocking, the plates were incubated with the sample for 1 h before being washed 3 times with TBST. Then, 100 µl of biotinylated 7A4 (b-7A4; Pierce NHS-biotinylation kit, as per manufacturer's

instructions) at 2 µg ml^{-1} in TBST was incubated for 1 h in each well, after which the plates were washed 3 times with TBST, and 100 µl of 1:500 dilution in TBST of 0.125 mg ml^{-1} strepavidin-conjugated horseradish peroxidase was incubated in each well for 1 h. The plates were washed 3 times in TBST, and 100 µl 2,2'-azino-bis (3-ethylbenzthiazoline-6 sulfonic acid), ABTS solution (200 µl 1% ABTS, 4.8 ml citrate buffer, 10 µl 30% H$_2$O$_2$) was added to each well. The optical density (OD) at 405 nm was read using a plate reader (Multiskan MCC EX, Thermo Electron Co.). Multiple readings were done, and the maximum rate of change in OD in each well was calculated. A non-linear regression was performed for each sample to find the least sum-of-squares fit for Eq. (1):

$$\text{rate} = \frac{R_{\max}}{1 + \left(\dfrac{C}{C_{50}}\right)^s} \qquad (1)$$

where 'rate' is the rate of OD change in each well, C is the sample concentration, $R_{\max}$ is the maximum rate, C_{50} is the concentration at which the rate is 50% of $R_{\max}$, and s is the slope coefficient. Since the concentration of mb-HSP70 at C_{50} was the same among samples, knowing the concentration of mb-HSP70 at C_{50} in the standard solution and the level of dilution at C_{50} for each of the samples allows the calculation of the concentration of mb-HSP70 in the undiluted sample (Ottinger et al. 2001).

Verifying specificity of an ELISA. We used ELISA to determine the concentration of mb-HSP70 in gonadal and somatic tissue, and in male and female clams to ensure that the mb-HSP70 was only present in female reproductive tissue. Ripe *Macoma balthica*, i.e. clams heavy with eggs or sperm, were collected from the York River on 23 October 2003, 30 September 2005, 30 October 2005, and 29 November 2005 and stored at –20°C before use. The clams were sexed (Gilbert 1978), and the following tissues were excised: (1) female somatic tissue from 3 clams (including samples of mantle, siphon, abductor muscle, foot muscle, and viscera); (2) female gonadal tissue (including eggs from 18 clams); and (3) male gonadal tissue from 6 clams. Each tissue sample was homogenized in PBS and centrifuged at 15 000 × g for 1 h at 4°C. The supernatant was collected and centrifuged a second time for further clarification at 15 000 × g for 1 h at 4°C, and the supernatant was collected. Protein concentration was determined for each extract using the BCA assay as above, and the extracts were stored at –20°C. The concentration of mb-HSP70 in each sample was determined by ELISA and standardized by dividing the concentration of mb-HSP70 by the total concentration of protein.

Quantification of eggs at different stages of gonadal development and in different years. In order to deter-

mine if the ELISA could be used to quantify eggs, we established the relationship between mb-HSP70 concentration and egg concentration for clams at different stages of gonadal development and in different years. Ripe female *Macoma balthica* were collected in the York River on 23 October 2003 (5 clams), 30 September 2005 (2 clams), 30 October 2005 (3 clams), and 29 November 2005 (8 clams) and stored at –20°C until used. Each clam was opened, its gonads incised, and samples of eggs extracted. Five clams were used from 23 October 2003, 2 from 30 September 2005, 3 from 30 October 2005, and 8 from 29 November 2005. The eggs were placed in PBS and gently suspended by repeated pipetting. The concentration of eggs in each sample was determined with a hemocytometer. *M. balthica* eggs are ~100 µm in diameter (Hendriks et al. 2003), so 15 µl of the egg suspension was placed on the hemocytometer; this was then covered with the cover slip and eggs were counted. We did 2 counts of each sample and took the average. Eggs could not be distinguished in clams sampled on 23 October 2003, so samples of the gonads were taken and analyzed with ELISA as below, but they were not included in the statistical analysis. The samples were centrifuged at 15 000 × g for 5 min, and the pellet was homogenized. The homogenates were centrifuged at 15 000 × g for 30 min, and the supernatant was collected and stored at –20°C. The concentration of mb-HSP70 in each sample was determined by ELISA. Total protein concentration was determined for each sample with the BCA assay as above, and the amount of protein per egg was calculated and statistically analyzed with a 1-way analysis of variance (ANOVA) with date (30 October 2005 and 29 November 2005) as the factor (all ANOVA-type analyses performed with Minitab v.15.1). The concentration of mb-HSP70 was regressed (linear, least-squares, all regression analyses were performed with SigmaPlot v.10.0) against the known concentration of eggs for each sampling period. The ratio of mb-HSP70:total protein (units mb-HSP70 mg^{-1} total protein) was calculated for the egg samples in October and November of 2005. These were compared to those calculated for the male gonadal and female somatic tissues (previous section) with a 1-way ANOVA and a Tukey's test. All samples were log(N + 10^{-4}) transformed to achieve homogeneity of variance prior to analysis.

Relationship between fecundity and BMI. Ripe female *Macoma balthica* collected on 30 October 2005 and 29 November 2005 were used to determine the relationship between fecundity and BMI. The shell length was measured with calipers to the nearest 0.1 mm, and the clams were opened and homogenized in 15 ml PBS. Homogenates were centrifuged at 3000 × g for 1 h, after which 1 ml of supernatant was

removed and the concentration of mb-HSP70 was determined by ELISA. The number of eggs in each clam was determined using the relationship previously determined between the concentration of mb-HSP70 and egg concentration. The rest of the sample was dried at 65°C and the dry mass was determined. The BMI (dry mass length^{-3} in mg cm^{-3}; Honkoop & Beukema 1997), also known as the condition index (Bonsdorff & Wenne 1989), was calculated for each clam. An analysis of covariance (ANCOVA) was conducted on egg number with date as a factor and BMI as a covariant. Linear regression was used to determine the relationship between the number of eggs in each clam and its BMI.

RESULTS

The ELISA was specific for female *Macoma balthica* gonadal tissue (Fig. 1). The non-linear regression was an excellent fit in every case, with r^2 values >0.98 and p values <0.0001. Small concentrations of mb-HSP70 were detected in female somatic tissues, but the con-
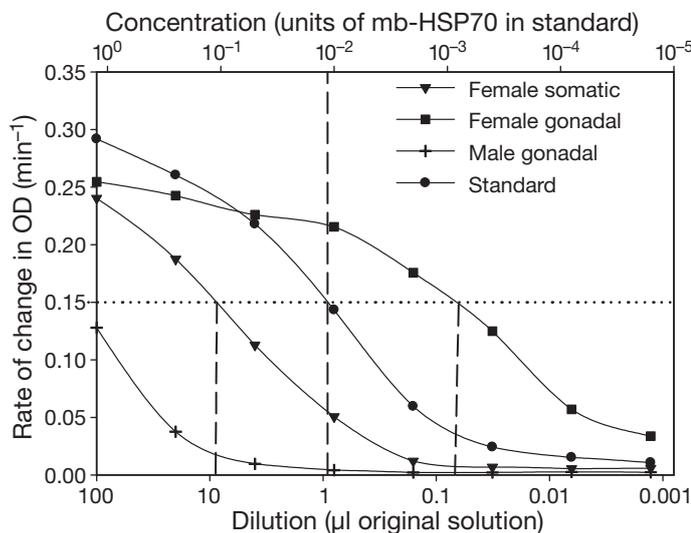

Fig. 1. *Macoma balthica*. ELISA curves for somatic and gonadal tissues from adult *M. balthica*. The standard line is a plot against both dilution (in µl) of the original solution (along with the other samples) as well as the concentration of mb-HSP70. The dotted horizontal line indicates the rate at C_{50} from the standard curve fit. The dashed vertical lines indicate the approximate C_{50} values for each curve based on the standard C_{50}. The C_{50} for each sample has the same concentration of mb-HSP70 as the C_{50} in the standard, so the dilution factor (in µl of the original sample) can be used to calculate the concentration of mb-HSP70 in the undiluted sample: standard (C_{50} = 1.0 µl, 1.25 units mb-HSP70 in undiluted sample), female somatic (C_{50} = 6.6 µl, 0.19 units mb-HSP70 in undiluted sample), female gonadal (C_{50} = 0.038 µl, 33.4 units mb-HSP70 in undiluted sample), male gonadal (unreliable fit, C_{50} = ~200 µl, ~0.006 units mb-HSP70 in undiluted sample)

centrations were 2 orders of magnitude lower than in female gonadal tissue (Fig. 1). Mb-HSP70 was below reliable detection limits in male gonads during all time periods, and our best estimate was 4 to 6 orders of magnitude lower than in female gonads. Western blot analysis (not shown) showed a weak reaction in the stacking gel layer of the polyacrylamide gel, and nothing at 96 kDa (the size of mb-HSP70, Bromage et al. 2008), indicating that the reaction is likely a non-specific binding of the antibody to large protein aggregates and that mb-HSP70 is not actually present in males.

Clams collected on 30 September 2005 were at an early stage of gonadal development; no eggs could be distinguished microscopically, and ELISA revealed extremely low levels of mb-HSP70 in the gonads, which were equal to the levels observed in male gonads and lower than those observed in female somatic tissue during gametogenesis. At all other time periods (23 October 2003, 30 October 2005, 29 November 2005), the mb-HSP70 concentration was positively correlated with the egg concentration (Fig. 2), although the relationship varied among the time periods. Clams collected on 29 November 2005 were fully mature (visual observation; Gilbert 1978); some had already spawned, as evidenced by empty gonads (Gilbert 1978). In 2005, total protein per egg increased from 0.016 µg egg^{-1} (±0.002 SE) in October to 0.030 µg egg^{-1} (±0.005 SE) in November (*t*-test; N = 27; *t* = –2.75; p = 0.012). The ratio of mb-HSP70:total protein differed among tissue types (Fig. 3; ANOVA; N = 40; $F_{4, 35}$ = 455.73; p < 0.0005) and was low in male gonads and in female gonads prior to egg development, intermediate in somatic tissue from ripe females, and highest in female gonads during egg development (Fig. 3).

The number of eggs in female *Macoma balthica*, ranged from 2500 to 450 000 eggs clam^{-1}, and was correlated to BMI (ANCOVA; N = 40; $F_{1, 38}$ = 11.82; p = 0.001), but not by month ($F_{1, 38}$ = 1.227; p = 0.276). Clams that had already spawned contained low levels of mb-HSP70, as expected, and were not included in the analyses. BMI was a good predictor of egg number, and clams with a BMI <1.4 mg cm^{-3} did not have any eggs (regression analysis; Fig. 4).

DISCUSSION

We have developed a simple and rapid assay for the quantification of eggs in female *Macoma balthica*. ELISAs are a highly quantitative, reliable means to measure concentrations of antigens (e.g. Choi et al. 1993). The ELISA developed in the present study has the added benefit of utilizing monoclonal antibodies, unlike other, similar assays that used polyclonal anti-
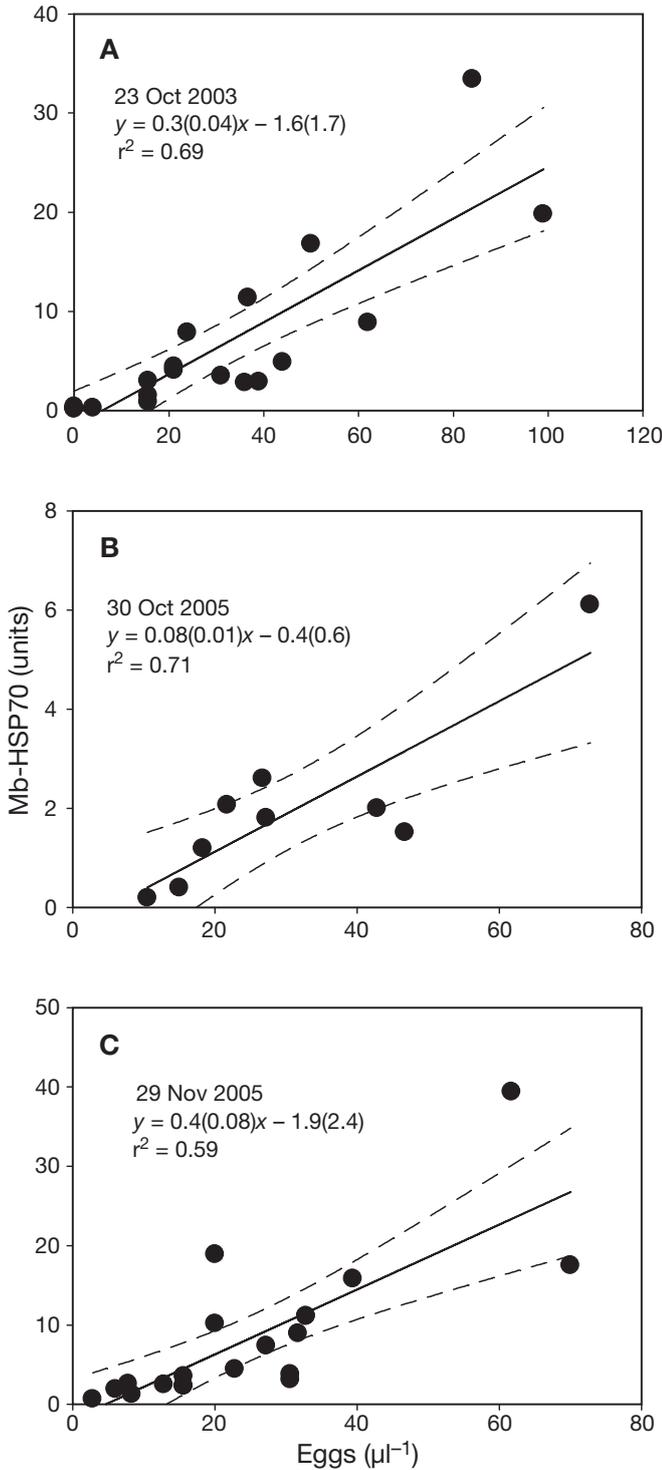
Fig. 2. *Macoma balthica.* Relationship between egg concentration and mb-HSP70 concentration at 3 different dates: (A) October 2003 (5 clams), (B) October 2005 (3 clams), and (C) November 2005 (8 clams). Equation and solid line represent a least-squares fit (p > 0.0003 for all 3 regressions; SE for regression parameters included parenthetically), and dashed lines represent the 95% confidence intervals for the regressions. Each point represents a sample of eggs taken from a female clam
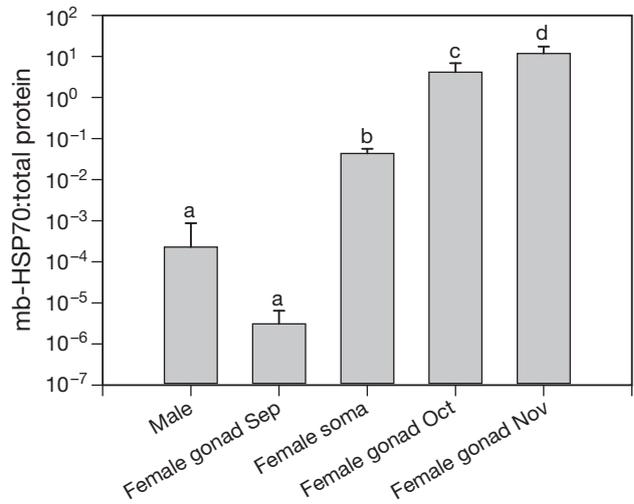

Fig. 3. *Macoma balthica.* Ratio (mean ± SD) of mb-HSP70:total protein in *M. balthica* tissues. Samples annotated with different letters differ at the 0.05 level (Tukey's test)

bodies to quantify bivalve eggs (Choi et al. 1993, Kang et al. 2003a,b, Park et al. 2003). Although polyclonal antibodies have similar utility in ELISAs, assays are only reliably comparable if they all use the same batch of antibodies. As polyclonal antibodies can only be produced in limited amounts, sharing between laboratories and comparison of results are likewise limited. This limitation does not exist with monoclonal antibodies; they are produced by an immortal hybridoma cell line that can be used in any laboratory to produce the same antibody, making direct comparison of results possible.

The ELISA in the present study is specific to egg tissue in female *Macoma balthica*. A small concentration of mb-HSP70 may be present in somatic tissues of ripe females, but this result could be due to the inclusion of a small amount of gonadal tissue in the sample, possibly due to leakage from the gonads during freezing and thawing or because gonads in ripe female *M. balthica* envelop most other tissues (Gilbert 1978), making dissection difficult. Regardless, the concentration of mb-HSP70 in these tissues is low enough that it does not influence the results. Male *M. balthica* and females prior to egg development had concentrations of mb-HSP70 below the reliable limit of this assay. This low level of reactivity in the ELISA, 6 to 7 orders of magnitude lower than that of egg tissue, was likely due to non-specific binding of the antibody to protein aggregates and not to the presence of mb-HSP70.

The concentration of the mb-HSP70 measured by this ELISA was linearly related to egg concentration in samples, and it increases with the maturity of the gonads. This necessitates calibration of the ELISA,
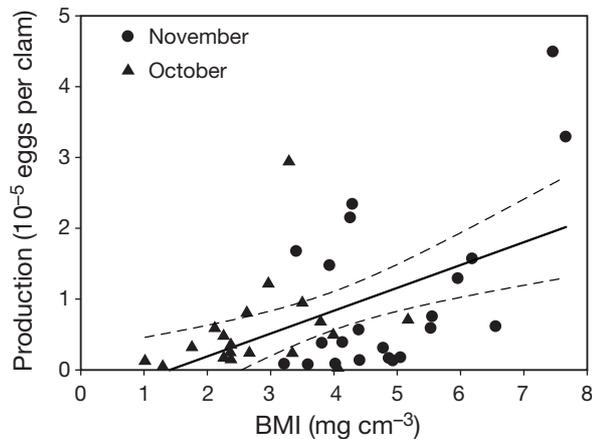
Fig. 4. *Macoma balthica.* Relationship between body mass index (BMI) and egg production in *M. balthica* in 2005. The solid line is the least-squares regression (p = 0.001; r^2 = 0.26; equation: eggs = 32 000 × BMI – 45 000), and dashed lines represent the 95 % confidence intervals for the regression

whenever it is used to quantify eggs, by the establishment of an egg concentration to mb-HSP70 concentration curve, as we do here. As this can be reliably done with 10 samples, it is not labor intensive. Without this calibration step, samples taken at the same time and from the same area may be compared with each other, but they cannot be compared with samples from another time or place.

The development of the gonads and eggs in *Macoma balthica* was similar to that observed in other studies (Shaw 1965, Gilbert 1978). Gonads began to develop in late September, though egg development could not be observed microscopically. Eggs developed between October and November, and spawning occurred in late November. This is later than reported by Shaw (1965), but, as spawning is temperature dependent and our samples were taken far south of those taken by Shaw, this is not surprising. The number of eggs in each clam did not change between October and November, but the total amount of protein in each egg increased, as well as the fraction of mb-HSP70 in that protein. This indicates that as soon as eggs are discernable under a light microscope, they increase in size, but not in number, so the assay can be used at any point during oogenesis without over- or under-estimating the fecundity of a given clam. In November, some clams had high BMIs, but few eggs. Many of these clams were small (16 to 19 mm), and some may have already partially spawned (Beukema et al. 2001), explaining the trend. Many potential roles of HSP70 in *M. balthica* could explain its increase with egg maturity. HSP70 proteins are involved in the folding of newly synthesized proteins and in the transport of proteins across membranes and, thus, are important during oogenesis (Billoud et al. 1993, Bukau & Horwich 1998). Additionally,

an HSP70, not expressed in non-reproductive adult tissues, is linked to meiosis during oogenesis and is essential in mitosis during embryogenesis in the sea urchin *Paracentrotus lividus* (Sconzo et al. 1999, Geraci et al. 2003).

The number of eggs in *Macoma balthica* from the Chesapeake increases with clam BMI, and clams with a BMI lower than ~1.4 mg cm^{-3} do not appear to invest energy in eggs. This is similar to *M. balthica* from the eastern North Atlantic, except that clams from the Chesapeake have a lower BMI (range in the Chesapeake: 1 to 8 [present study]; range in the eastern North Atlantic: 4 to 14 [Beukema et al. 2001]) and are likewise capable of reproduction at a lower BMI (1.4 in the Chesapeake [present study]; 5.5 in the eastern North Atlantic [Honkoop et al. 1999]). This is likely due to differences in growth patterns; *M. balthica* in Europe are wider and broader than those of a similar length in the Chesapeake (Beukema & Meehan 1985) and have a greater mass (Kamermans et al. 1999), which would result in a lower BMI (they may indeed be a different species; Väinölä 2003). However, *M. balthica* in both areas have a similar number of eggs at the same size. Our smallest fecund clams (16 to 19 mm shell length) had between 7000 and 60 000 eggs, which is similar to 15 mm clams from the Wadden Sea that had between 10 000 and 90 000 eggs (Honkoop & van der Meer 1998). Many of the clams that had high BMIs but had fewer eggs than expected were these small clams.

CONCLUSIONS

We present here a new method for quantifying eggs in *Macoma balthica.* Our technique is accurate, simpler, and cheaper to perform than other methods, and will allow for meaningful comparisons among clams from different systems. This method has been tested thus far only on clams from Chesapeake Bay and is likely applicable to all western North Atlantic populations of *M. balthica.* As genetic differences exist between the western and eastern populations of *M. balthica* (Meehan 1985, Väinölä 2003), the assay still needs to be verified for clams from eastern North Atlantic populations.

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