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Thomas Knigge, Fatima Dahboul, Damien Alain, Tiphaine Monsinjon. The gametogenic cycle and oestradiol levels in the zebra mussel *Dreissena polymorpha*: a 1-year study. *Journal of Molluscan Studies*, 2015, 81 (1), pp.58-65. 10.1093/mollus/eyu056 . hal-04699774

**HAL Id: hal-04699774**

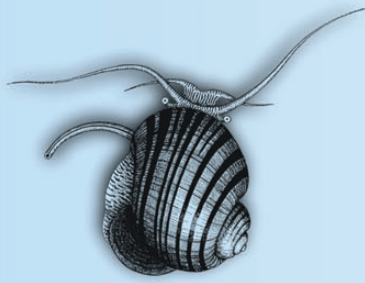
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## The gametogenic cycle and oestradiol levels in the zebra mussel *Dreissena polymorpha*: a 1-year study

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(Received 17 February 2014; accepted 11 June 2014)

### ABSTRACT

As a common sessile filter feeder with high bioconcentration rates, *Dreissena polymorpha* is useful as a bioindicator species. Pollution and environmental factors influence the reproductive effort of these bivalves. Therefore, the evaluation of the gametogenic cycle is important for understanding environmentally-related physiological alterations in this dioecious species. Animals from an unpolluted reference site close to the highly polluted Seine River, France, were monitored monthly over 1 year using qualitative and quantitative measures of gametogenesis. Developmental stages of the gonad were determined by visual inspection of histological slides. Unbiased quantitative stereological analysis was conducted to estimate oocyte development and the gonad index was determined. To investigate a possible role of female sex steroids in bivalve gametogenesis as a possible target for xenoestrogens, free oestradiol levels were assessed and analysed with respect to oocyte development. The basic pattern of the reproductive cycle consisted of early gametogenesis during autumn and winter, followed by rapid vitellogenesis in early spring, and spawning towards the summer months. However, the population was characterized by high inter-individual variability of the gonad stages. Furthermore, the simultaneous occurrence of different developmental stages within the same animal made classification difficult. Stereological determination of oocyte volumes as well as gonadosomatic index provided a more precise evaluation of reproductive effort. Oestradiol levels did vary significantly between months, but did not show any clear relationship with a particular stage of gametogenesis or oocyte volume.

### INTRODUCTION

Zebra mussels, *Dreissena polymorpha* (Pallas, 1771), are widespread and abundant freshwater molluscs that can be found in many lakes and rivers of Europe and North America (Jantz & Neumann, 1998). Because it is an invasive species that threatens the native unionid mussel populations, it is mainly considered an ecological menace. On the other hand, it has become an important component of freshwater ecosystems with positive effects on water quality due to the enormous filtration activity of large populations of zebra mussels (Smit *et al.*, 1993). This filtering capacity also renders zebra mussels a good biomonitor, because they concentrate pollutants (Bervoets *et al.*, 2004; Minier *et al.*, 2006). For this reason, as well as for its abundance, zebra mussels have been the subject of numerous ecotoxicological studies (Smolders *et al.*, 2004; Peck *et al.*, 2007; Palais *et al.*, 2011). There is a large body of literature that examines the ecology and reproductive biology of *Dreissena* species (e.g. Morton 1969a, b, c; Borcharding, 1991; McMahon, 1996; Nichols, 1996; Ram, Fong & Garton, 1996; Jantz & Neumann, 1998; Wacker & von Elert, 2003; Alexander & McMahon, 2004). Their abundance and a relatively comprehensive

knowledge of their gametogenic cycle and spawning behaviour renders the zebra mussel a suitable organism in which to study possible endocrine-disrupting effects in bivalves (Hardege, Duncan & Ram, 1997). Sex and reproductive state are of fundamental importance for the evaluation of pollution-caused stress in *D. polymorpha*, which adds to natural stressors affecting the general health and reproductive status of the animals (e.g. Palais *et al.*, 2011). Global warming may also affect the gametogenic cycle. For instance, spawning in *D. polymorpha* appears to be related to temperature, and the allocation of energy to reproduction may be reduced when the water becomes too warm and catabolism exceeds assimilation rates (Waltz, 1978; Wacker & von Elert, 2003; Alexander & McMahon, 2004).

Nonhistological examination of gametogenesis is frequently done by determination of the gonadosomatic index (GSI), i.e. the gonadal wet weight as a proportion of the total soft body wet weight. However, in *Dreissena* species and many other bivalves, the gonad and digestive gland are intertwined. Thus, an accurate determination of the GSI is difficult and may require sophisticated morphometric methods (Borcharding, 1992) to determine gonad volume in relation to that of the somatic tissues. Determinations of the gametogenic cycle and gonad

maturity in bivalves are usually carried out by visual examination of histological sections (e.g. Garton & Haag, 1993; Jubel *et al.*, 2003; Palais *et al.*, 2011). This procedure requires profound knowledge of the structural characteristics defining the different developmental phases (e.g. Eckelbarger & Davis, 1996) and experience in the interpretation of the histological images. Nevertheless, the discrimination of stages is set arbitrarily (MacDonald & Thompson, 1986) and the categories differ in the literature (Heffernan, Walker & Carr, 1989; Garton & Haag, 1993; Alfaro, Jeffs & Hooker, 2003). In addition, the gonad usually does not develop in a completely uniform manner (Lenoir & Mathieu, 1986; Neuman, Borcharding & Jantz, 1993); different stages may be present in adjoining areas of the same gonad, which may complicate the decision to assign an individual to a distinct gonadal developmental stage. Hence, visual examination is highly subjective. The judgement made by the analyst may also be influenced by prior knowledge, which could bias the results towards the stages expected for a certain period of the year. Therefore, determination of gonadal maturity stages using qualitative histological examination must be considered an inaccurate method to determine the maturity of the gonad (Najmudeen, 2008). As an alternative, quantitative methods such as planimetry (Kennedy & Battle, 1964) have been proposed. A measure that has been employed frequently is the determination of the oocyte diameters (e.g. Brown, 1984; Neuman *et al.*, 1993). This is complicated by the fact that when cutting through a spherical structure, the diameter varies with the position of the section through the sphere. Brown (1984) circumvented this problem by only considering oocytes cut through the nucleus, but since the proportion of the nucleus to entire oocyte changes during vitellogenesis, this criterion could be a source of variability. Therefore, various authors have used stereological methods as a means of continuous and unambiguous determination of gonad maturity (e.g. Lowe, Moore & Bayne, 1982; Newell *et al.*, 1982; MacDonald & Thompson, 1986; Borrero, 1987; Morvan & Ansell, 1988; Navarro, Iglesias & Larrañaga, 1989). To this end, numbers of oocytes as well as the relative volume occupied by the oocytes within the gonad are quantified (Morvan & Ansell, 1988; Heffernan *et al.*, 1989; Borcharding, 1991). According to the stereological literature, however, establishing numbers and measuring diameters or surfaces requires procedures much more complex and laborious than simple point counting (Gundersen *et al.*, 1988b). Point counting stereology has been used previously by several authors for the study of the gametogenic cycle in molluscs (Bayne *et al.*, 1978; Lowe *et al.*, 1982; Newell *et al.*, 1982; Lubet *et al.*, 1991). However, these methods seem to have lost favour with the development of computer-assisted image analysis (e.g. Heffernan *et al.*, 1989; Meneghetti, Moschino & Da Ros, 2004). Nevertheless, stereological methods are widely applied in medical research and remain a serious alternative to many qualitative staging methods, providing a truly quantitative measure of gametogenesis (Alvarez Fariña & Gual-Arnau, 2006).

The present study aimed to investigate methods of assessing gametogenetic development of the gonad in *D. polymorpha* throughout a 1-year reproductive cycle. The objective was to conduct a comparative study on different parameters of gonad development to see how these parameters would allow for the observation of vitellogenesis. To this end, mussels were collected monthly at a largely pristine site. Gametogenesis was assessed qualitatively by means of histology, and point-counting stereology was employed as a quantitative method to measure oocyte volumes on a continuous scale as opposed to the discrete oocyte developmental stages. Furthermore, the GSI was determined by separating gonadal tissue from the digestive gland and the rest of the somatic tissue. By doing so, gonadal tissue was retained as material for measuring free oestrogen levels in order to examine

the possible effect of this female sex steroid hormone on the bivalve gametogenic cycle. Several studies have suggested that vertebrate sex steroids play a role in sexual determination and maturation of bivalves and other molluscs, although the mechanistic basis for such a role remains largely unknown. For instance, 17 $\beta$ -oestradiol (E2) was used to direct the sex ratio in *Crassostrea gigas* towards a higher percentage of females (Mori, 1982). E2 was also considered to promote vitellogenesis in the scallop *Patinopecten yessoensis* (Osada *et al.*, 2003) and to increase the number of offspring in the mudsnail *Potamopyrgus antipodarum* (Stange *et al.*, 2012). Consequently, a number of studies have investigated the potential of endocrine-disrupting chemicals to interfere with molluscan reproduction via pathways similar to that of vertebrates (e.g. Quinn *et al.*, 2004; Lazzara *et al.*, 2012; Stange *et al.*, 2012; Zhang, Pan & Zhang, 2012). Some authors also related changes in oestrogen levels to the bivalve gametogenic cycle (Gauthier-Clerc, Pellerin & Amiard, 2006; Ni, Zeng & Ke, 2013). This, however, depends in part on how the gametogenic cycle is determined and whether it consists of discontinuous classes of gonad maturation or a continuous measure of gonad or gamete development. Therefore, in this study, both types of measures were related to free oestradiol levels, as this represents the potentially active form of the female sex steroid, which could act on gonadal maturation.

## MATERIAL AND METHODS

### Sampling

Mussels were collected monthly from February 2005 to May 2006 at a quarry pond close to Yville-sur-Seine, France (00°53'01"E, 49°25'54"N). The specimens were removed by hand from floating jetties within the upper 1 m depth. Water temperature was recorded at each sampling event. The mussels were transported to the laboratory in aerated lake water at ambient temperature and care was taken to avoid temperature deviation with respect to the natural site. For each sampling, 60 mussels of 18–29 mm length ( $22.61 \pm 2.06$ ) were dissected by cutting the posterior adductor muscle and removing the visceral mass. The gonad of both males and females consists of a Y-shaped organ, the two-paired branches of which lie over the digestive gland (DG). Gonadal and DG tissues were meticulously separated on an ice-cold surface using a stereomicroscope. Total soft body and gonadal tissue were weighed with an analytical balance to the closest mg for determination of the GSI, calculated as  $(\text{GWW}/\text{TWW}) \times 100$ , where GWW = gonadal wet weight and TWW = total wet weight (Çek & Şereflişan, 2006). The posterior tip of the gonad, which is not connected to DG tissue, was excised and fixed at room temperature in formaldehyde (4% w/v buffered at pH 6.9; Carlo Erba reagents, Rodano, Italy). The remaining gonadal tissue was snap-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  for further analysis.

### Histology and stereology

Gonad samples were dehydrated in an ascending alcohol series (70, 90 and 100%) and placed in Paraplast embedding medium without any specific orientation, since preliminary observations revealed structural homogeneity of the gonadal tissue. Sections 4- $\mu\text{m}$  thick were cut using a rotary microtome (Leica RM 2125 RT, Leica Microsystems, Nussloch, Germany) and stained with haematoxylin and eosin, following standard procedures. Microscopic observation of the slides allowed for sex determination as well as the determination of gametogenic stages according to Jubel *et al.* (2003): stage 0, resting stage; stage 1, early development; stage 2, late development/ripe; stage 3, spawning and stage 4, reabsorbing. Female specimens ( $n = 10\text{--}29$ ) were further analysed by stereology to enable an unbiased

quantitative and continuous measure of oocyte size. Point counting of oocytes was conducted according to Weibel & Elias (1967) using micrographs of the 4- $\mu\text{m}$  tissue sections taken with Q-win software (Leica Microsystems Imaging Solutions, Cambridge, UK). A graticule overlay adapted to the size range of the oocytes (B36 square lattice test system, with 144 test points used at a magnification of 400 $\times$ ) was taken from Weibel (1979). The volume density of oocytes within the gonadal tissue was assessed for randomly selected areas on five different serial sections per individual. Intersections (points) that fell on the oocytes were counted. Volume fractions of oocytes ( $\text{oV}_v$ ) were calculated by dividing the number of points tallied (P) by the total number of test points (T):  $\text{oV}_v = P/T$ . Only oocytes clearly identifiable as such, i.e. from early vitellogenic to mature oocytes, were taken into account, since oogonia and primary oocytes bear the risk of being smaller than the section thickness of 4  $\mu\text{m}$  and may be confounded with other cytological structures. The volume of the reference space, i.e. the gonad itself, was estimated by its weight, assuming a specific gravity of 1 (Gundersen *et al.*, 1988a) to account for changes in the size of the organ. Thus, proportional volume fraction data calculated by stereological equations as percentages were multiplied by the mass of the reference space and converted into  $\text{cm}^3$  of total oocyte volume ( $V_{\text{tot}}$ ).

### Steroid hormone analysis

Mussels from a separate batch dedicated to the analysis of steroid hormone were used for extraction of oestradiol and the specimens were selected according to month and developmental stage, respectively, in order to obtain specimens that were representative of the stages prevalent in each month of a complete gametogenic cycle, following the criteria of Juhel *et al.* (2003). Subsequently, the  $\text{oV}_v$  for these mussels were determined for

correlation with oestrogen levels. Free oestrogen (E2) was estimated as described previously by Gauthier-Clerc *et al.* (2006). Individual gonad samples (10–100 mg; average wet weight  $52.96 \pm 22.49$  mg) were homogenized and sonicated in 500  $\mu\text{l}$  of water. Due to unequal sex distributions and/or technical problems, the sample size varied between 2 and 11 individuals per month and stage; no sample could be analysed for May 2005. The homogenates were digested with 400  $\mu\text{l}$  25 mM HCl for 15 min at 40  $^\circ\text{C}$ , after which 1.25 ml 0.07 M  $\text{Na}_2\text{HPO}_4$  (pH 7.4) was added. Organic extraction was carried out twice using 14 ml dichloromethane, followed by subsequent evaporation of the solvent under nitrogen. Extracts were dissolved in 250  $\mu\text{l}$  assay buffer provided by the manufacturer. Enzyme immunoassay for E2 detection (100% E2 specificity, detection limit 8 pg/ml; Cayman Chemical Co., Ann Arbor, MI, USA) was carried out following the manufacturer's specifications. The measurements were corrected for the differences in wet weight of the gonad samples. Additionally, some male gonads were analysed to check for possible differences in E2 levels between the sexes.

### Statistical procedures

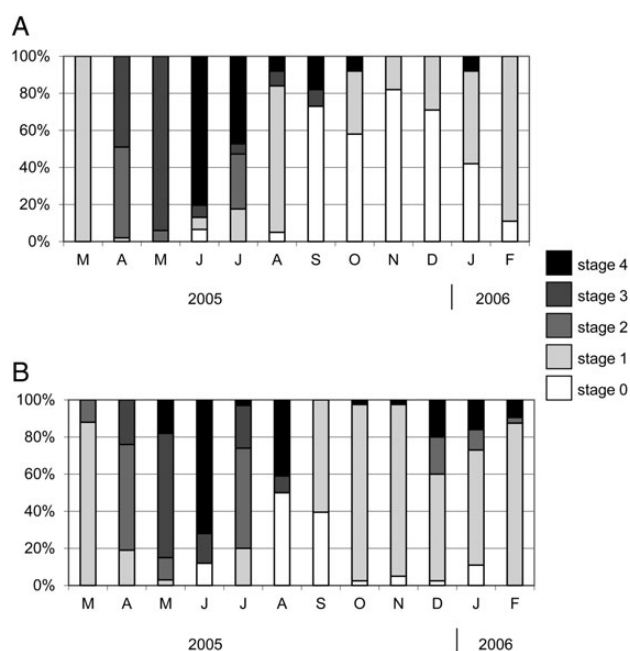
As  $\text{oV}_v$  essentially reports proportional data, the majority of which are below 30%, arcsine transformation was carried out to normalize  $V_{\text{tot}}$  prior to pairwise comparison with Student's *t*-test (Coward & Bromage, 2002). The proportional GSI values showed a similar distribution range and were treated in the same way as the stereology data. (It should be noted, however, that in order to enhance comprehension and interpretability, the figures depict back-transformed values.)

For all other statistical analyses, measures were calculated as means  $\pm$  standard deviation. Free oestradiol levels were subjected to nonparametric Kruskal–Wallis analysis of ranks and pairwise Wilcoxon *U*-tests. Data for  $\text{oV}_v$  and free E2 levels were subjected to nonparametric Spearman's rank-order as well as Pearson product-moment correlation. All statistical operations were run on STATISTICA v. 9 (StatSoft, Tulsa, USA).

## RESULTS

### Qualitative histological analysis

Visual examination of the histological sections of the gametogenic cycle for the entire sample of individuals revealed the following features (Fig. 1): (1) a highly coordinated phase of early development with *c.* 90% of males and females being in stage 1 at the end of winter (March 2005, February 2006); followed by (2) a phase of germ-cell maturation into either spermatozooids or secondary oocytes during April, in which gonad development remained quite synchronized for many of the males and females, i.e. half of the males and females being in stage 2; (3) a spawning period, which culminated in May, in which a large number of gametes were released given that almost 70% of the females and nearly all males were found to be in stage 3; (4) a period of tissue reorganization or rearrangement of the gonad, resulting either in maturation of further cohorts of spermatozooids and oocytes for subsequent spawning in some of the individuals, or reabsorption of germ cells with entry into the resting stage for others. Our observation of histological sections of gonads revealed a remarkably diverse developmental pattern of the gonadal tissue during the summer months, both for males and females, most significantly for the latter. There was some indication of a second spawning in midsummer, since about 30% of the males and 50% of the females were found to be in stage 2 in July. No fully synchronized event, however, could be inferred from the histological analysis. Gametogenesis obviously may have continued until the beginning of autumn so that only part of the



**Figure 1.** Changes in gonadal stages from male (A) and female (B) *Dreissena polymorpha* throughout a 1-year reproductive cycle from March 2005 to February 2006, as determined by histological observation following the method of Juhel *et al.* (2003). Stage 0, resting; stage 1, early development; stage 2, late development/ripe; stage 3, spawning; stage 4, reabsorbing. The bars show percentages of each stage over the entire sample per month (A,  $n = 26-36$ ; B,  $n = 25-34$ ).

population showed preponderant reduction of the acini with reabsorption of germ cells in favour of vesicular connective as well as adipogranular tissue. Hence, a clearly marked resting period could not be identified. Also, individual mussels could be effectively and unambiguously sexed in the majority of cases, either by virtue of ongoing germ-cell development, or because of the presence of residual sperm cells or oocytes. Eventually, the population entered (5) early gametogenesis in autumn, a phase characterized by a fairly heterogeneous development, in particular between males and females. During this autumnal phase of early development, oogenesis advanced considerably in large parts of the female gonads, leading to 20% of stage 2, i.e. late development/ripe individuals at the beginning of winter (Fig. 1B) as well as 20% stage 3, i.e. showing phenomena of reabsorption, albeit not associated with disintegration of the gonadal tissue as in late summer.

### Temperature

The temperature profile obtained for the quarry pond for March to December 2005 (Fig. 2) was typical of the annual temperature cycle of lakes of temperate zones. The temperature profile showed a continuous increase of surface temperature from 11 °C until the maximum of 25 °C in June, staying at this level throughout the summer, followed by a gradual decline during autumn to reach a low of 6 °C in December, which was, however, still slightly above being isothermal. The 4 °C minimum was reached in January and February 2006, allowing for overturn that results in uniform distribution of oxygen and nutrients from top to bottom. After March 2006, temperatures began to rise similarly to 2005, but stayed at somewhat lower levels until the end of recording in May 2006.

### Gonadosomatic index

The GSI of males and females followed a similar course, although the male GSI tended to be generally somewhat lower than that of females ( $\Delta$ GSI 1–3%; data not shown), notably after spawning in May 2005 and 2006 ( $\Delta$ GSI, 3%;  $P = 0.0394$  and  $\Delta$ GSI, 1%;  $P < 0.0001$ , respectively) as well as from September to November ( $\Delta$ GSI, 1% for all months;  $P = 0.0041$ ,

$P = 0.0231$  and  $P = 0.0153$ , respectively). In July 2005 the small peak present in females with a mean GSI of 19%, suggesting renewed growth of the female gonads, was absent in males (12%;  $P < 0.0001$ ). Hence, for clarity and simplification in the following, we refer only to the female GSI as depicted in Figure 2.

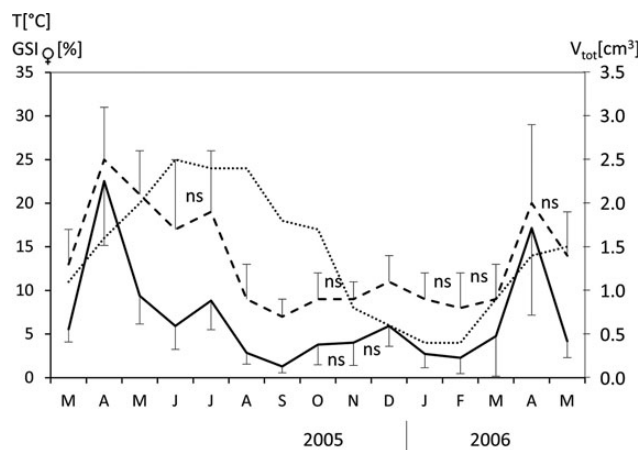
The overall changes in the GSI corresponded closely with the histological observations in terms of gonadal developmental stages described above. Early development (stage 1) was accompanied by an intensive growth leading to a significant peak in April ( $P < 0.0001$  and  $P = 0.0029$  as compared with March and May, respectively) when gonads were either in late development or already ripe. The rapid decline in the GSI continued from May to June ( $P = 0.0085$ ), which was in line with the spawning stage that predominated for these months. In July, the observation of newly late-developmental or ripe gonads for more than 50% of the females was apparently reflected in a slight, but not significant, increase in the GSI ( $P = 0.1791$ ), which was again followed by a sharp drop in August ( $P < 0.0001$ ), suggesting a second spawning event. The first low was reached in September and was followed by an immediate increase in the GSI, both alterations being significant ( $P = 0.0416$  and  $P = 0.0014$ , respectively). This almost imperceptible growth, characterized by only slightly elevated GSIs, continued in the following months and resulted in a small, but significant, peak in December ( $P = 0.0014$  as compared with November). Subsequently, the GSI declined from December to January ( $P = 0.0008$ ), presumably corresponding to the reabsorption of nearly mature oocytes already developed at this time, and remained unchanged thereafter ( $P = 0.2045$  for January to February and  $P = 0.0983$  for February to March) until it rose steeply towards its April maximum ( $P < 0.0001$ ), which was less strongly developed than in April 2005.

### Stereological analysis

The means for the total oocyte volume mirrored closely the changes observed for the female GSI (Fig. 2). Total oocyte volume increased rapidly from March to April ( $P < 0.0001$ ) and in June returned to values recorded prior to this climax ( $P < 0.0001$ ). From June to July  $V_{\text{tot}}$  again rose to reach a minor, but significant peak ( $P = 0.0005$ ), from which it declined to its lowest point in September ( $< 0.005 \text{ cm}^3$ ;  $P < 0.0001$ ). At the beginning of autumn a slight but significant increase of  $V_{\text{tot}}$  was observed ( $P = 0.001$ ). From the small peak in December the oocyte volume declined again until February ( $P = 0.003$ ), but was not as low as it was in September. Afterwards,  $V_{\text{tot}}$  first increased slightly, but surged between March and April ( $P < 0.0001$ ). The maximum volume occupied by the oocytes in April 2006, however, was not as large as that of 2005.

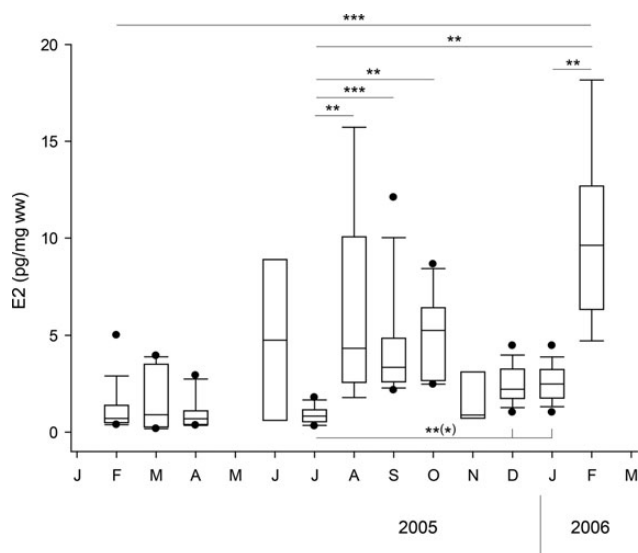
### Free oestradiol

Female E2 levels did not seem to follow an obvious pattern with any of the developmental stages (Fig. 3). Specific months, like August 2005 and February 2006, were characterized by a high variability of E2 levels. Other months, i.e. June and November 2005, were not sufficiently represented by measurement values or not represented at all in the case of May. Nonetheless, statistically significant differences in E2 levels of female gonads were obtained over all groups ( $P < 0.0001$ ). The first half of 2005 was characterized by low E2 levels from February to July, with the differences between the samples being nonsignificant. In late summer and autumn 2005 a significant rise in E2 levels could be observed ( $P = 0.0025$ ,  $P = 0.0003$  and  $P = 0.0012$  for July vs August, September and October, respectively). With the exception of the unreliable mean for November, E2 levels also stayed higher in the following months as compared with those of the

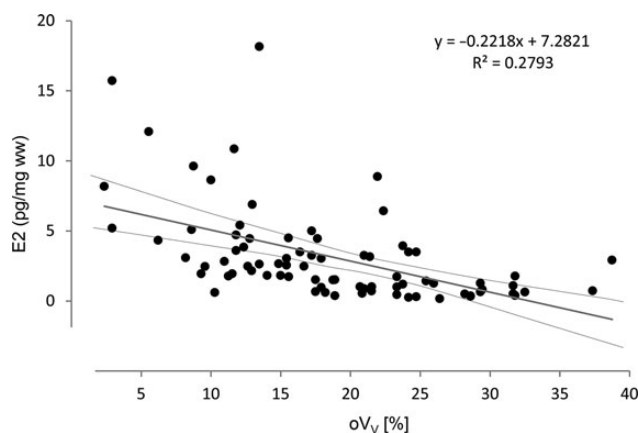


**Figure 2.** Changes in the gonadosomatic index (GSI, dashed line), temperature ( $T$ , at 0.5 m depth, dotted line) and total oocyte volume ( $V_{\text{tot}}$ , continuous line) for female individuals of *Dreissena polymorpha* collected from March 2005 to March 2006. Means and SD,  $n = 21$ –35 for GSI and  $n = 10$ –29 for  $V_{\text{tot}}$ . All month-to-month changes of GSI and  $V_{\text{tot}}$  significantly different at  $P \leq 0.05$ , except where indicated as nonsignificant (ns).

first half of 2005 ( $P = 0.0012$ ,  $P = 0.0008$  and  $P = 0.0025$  for July vs December, January and February, respectively). However, E2 levels in December 2005 and January 2006 were lower than those during August to September 2005 ( $P = 0.0434$  and  $P = 0.0225$  for September and August vs December as well as  $P = 0.0027$  between October and January). Notably, the increase observed from December 2005 to February 2006 ran just ahead of the pronounced increases of  $V_{\text{tot}}$  and GSI in 2006. Thus, when plotting oestradiol levels against oocyte volume density, a moderately negative correlation was obtained (Spearman  $\rho = -0.63$ , Pearson  $R = -0.53$ ; both  $P < 0.0001$ ; Fig. 4). Consequently, higher E2 levels corresponded to low oocyte volume density, whereas low oestradiol levels corresponded



**Figure 3.** Free oestradiol (E2) levels in female *Dreissena polymorpha* during a 1-year reproductive cycle from February 2005 to February 2006. The central line represents the median E2 level for each month, the box the interquartile range between the first and third quartiles and the whiskers (error bars) above and below the box indicate the 90th and 10th percentiles; outliers are plotted as individual points. Sample size ( $n$  per month): Feb 05 = 11, Mar 05 = 6, Apr 05 = 6, May 05 = 0, June 05 = 2, July 05 = 7, Aug 05 = 5, Sept 05 = 9, Oct 05 = 6, Nov 05 = 3, Dec 05 = 10, Jan 06 = 11, Feb 06 = 5;  $\alpha$ -levels defined as significantly different for  $0.001 < P \leq 0.01$  (\*\*) and highly significant for  $P \leq 0.001$  (\*\*\*).



**Figure 4.** Pearson correlation for free oestradiol (E2) levels and oocyte volume fraction ( $oV_v$ ) in gonads of *Dreissena polymorpha*;  $n = 80$ ,  $R = -0.53$ ,  $P < 0.0001$ ; 95% confidence limits.

to the periods when oocytes occupied large parts of the gonad, i.e. when they were well developed.

## DISCUSSION

The aim of this study was to follow the gametogenic cycle of an important bioindicator species, *Dreissena polymorpha*, which can be found in many polluted surface waters. Growth and reproduction are ecologically relevant endpoints to assess the acute and chronic effects of pollution, and therefore evaluation of sexual maturation represents an important parameter to be taken into consideration in the face of xenobiotic stressors (Jäger *et al.*, 2006; Palais *et al.*, 2011). Zebra mussels show an astonishing physiological plasticity and may adapt their reproductive cycles according to varying environmental factors (Nichols, 1996). Consequently, reproductive cycles for *D. polymorpha* described in the literature (Borcherding, 1991; Garton & Haag, 1992; Ram *et al.*, 1996; Gist, Miller & Brencce, 1997; Jantz & Neumann, 1998; Juhel *et al.*, 2003) revealed high variability, depending on location, water depth, year or region (Neumann *et al.*, 1993; Nichols, 1996). Notwithstanding a wide consensus on the principal qualitative aspects of gonad development in this and other bivalves, a precise prognosis of the timing of specific gametogenic events for a particular population is difficult to achieve (Nichols, 1996). Therefore we attempted to characterize the gametogenic cycle in a defined reference population close to the highly polluted Seine Estuary (Minier *et al.*, 2006) more accurately than has been done previously (Peck *et al.*, 2007). The methods were evaluated for their capacity to specify the precise stage of gonadal development on the individual level.

The fundamental characteristics of the gametogenic cycle of *D. polymorpha* in central and northwestern Europe can be confirmed by qualitative histological observations using the staging method of Juhel *et al.* (2003). With this qualitative evaluation, it is possible to identify a broad synchrony of early development towards the end of winter followed by a rapid maturation, indicative of vitellogenesis, and spawning in May to June. According to the literature the spawning period appears to be somewhat variable and depends on the water depth and the corresponding temperature as well as on the region (Borcherding, 1991, 1992; Haag & Garton, 1992; Neumann *et al.*, 1993; Gist *et al.*, 1997). The resting period in late summer could also be detected, although less clearly, and corresponded to those reported by Borcherding (1991, 1992) at water depths equivalent to those in our study. Although the qualitative nature of the reproductive cycle may be described for an entire population, if the sample size is sufficiently large, determination of gonadal maturity on the individual level may be inaccurate. In particular, the determination of the individual gonad stage of females remains ambiguous due to the development of successive cohorts of oocytes (Neumann *et al.*, 1993; Gist *et al.*, 1997). In fact, different developmental stages can often be observed in the gonad of the same individual. It must be assumed that in this case the classification of individuals into a distinct developmental stage will be subjective, and that it may require secondary information such as sampling date to attribute an individual to the correct stage. This alleged bias may thus produce—and perhaps reproduce—a general picture of the overall development of gonad maturity for an entire population. However, it is likely to underestimate phenotypic plasticity of reproduction within the population.

A nonhistological quantitative method that is generally easy to implement and often used for the evaluation of gonad development is the GSI (Lubet *et al.*, 1991; Pazos *et al.*, 1996; Çek & Şereflişan, 2006). Because of the intertwined gonad and digestive gland in many molluscs, however, this becomes a challenge. The determination of the volume of the gonad in relation to the volume of other somatic tissues using serial sections across the

entire organ may be a more accurate method for obtaining a GSI in a species such as *D. polymorpha* (Borcherding, 1992). However, if it is necessary to use gonad and/or other tissue from the same individual in order to examine other parameters, morphometry is not possible. In this case this method can only provide a general gametogenic status for the population. Phenotypic anchoring to the individuals that serve for other analyses, however, will be impossible. Therefore, we aimed at separating the gonadal tissue from the somatic tissue. Since the colour and the structure of gonadal tissue are different from that of the digestive gland, the tissues can be pulled apart from each other and separated. If this is done thoroughly, the GSI accurately reflects the gonad growth, displaying the major gametogenic events. The GSI is thus a feasible method to establish a quantitative measure of gonad development both for females and males, even in molluscs with intertwined organs. Nevertheless, the separation of gonads from other tissue is time-consuming and laborious. Moreover, the GSI by itself does not inform directly on the sex and the maturity of the oocytes/spermatozooids, so that it may need to be accompanied by some qualitative analysis.

A particular advantage of stereology as a histological method of establishing gonadal maturation is that it removes possible bias on the part of the investigator, a substantial drawback of staging methods, and provides a reliable means of comparing histological data. We believe that this represents an indispensable prerequisite to attribute a certain developmental stage to each particular individual. However, as pointed out by Hilbish & Zimmermann (1988), oocyte volume fraction provides a measure of the relative maturity but not of the actual reproductive condition, unless it is related to changes of the total volume or weight of the gonad (see also Gundersen *et al.*, 1988a). Moreover, to be meaningful, stereology should generally focus on absolute instead of relative values (Gundersen *et al.*, 1988a; Mouton, 2002). Since it was not possible to estimate the true volume of the gonad using the Cavalieri principle (Gundersen *et al.*, 1988a; Mouton, 2002; see also the morphometrical method of Borcherding, 1992), we had to rely on the gonad weight as an estimate (Mouton, 2002). Although the possibility of changes in specific gravity with varying lipid contents of the gonad tissue or that of variable shrinking following the histological processing of biological tissue cannot be ruled out completely (Mouton, 2002), the values for  $V_{\text{tot}}$  must be considered the most pertinent for assessing the individual state of gonad maturation, at least as far as females are concerned. However, the method is not applicable to males since the developing sperm cells are too small to be analysed by point counting with light microscopy.

As an application of the point counting estimate of oocyte volumes, we sought to relate gametogenesis in females to free E2 in the gonadal tissue. Numerous studies have demonstrated oestrogens to be present in tissues of bivalve molluscs (e.g. Reis-Henrique *et al.*, 1990; Gauthier-Clerc *et al.*, 2006; Peck *et al.*, 2007). Although Peck *et al.* (2007) estimated the sampling site to be essentially free of contamination with xenoestrogens, given that concentrations in water and sediment samples were below the detection limit of the Yeast Estrogen Screen (YES) assay (Routledge & Sumpter, 1996), i.e. below  $0.01 \text{ ng l}^{-1}$  for the water column, it cannot be ruled out that the mussels accumulated E2 from the aquatic environment. The quarry pond from which the mussels were sampled is situated alongside the Seine River and is surrounded by settlements and farmland, so that an entry of steroid hormones excreted from vertebrates or by phreatic waters cannot be excluded. Bioconcentration rates of E2 by the zebra mussel have been reported to be several hundredfold (Peck *et al.*, 2007), so that it is conceivable that the mussels may contain detectable amounts of E2, even if concentrations in the environment were below detection limits.

Generally, most of the E2 in molluscs is found to be stored in the form of biologically inactive fatty acid conjugates (Janer *et al.*, 2006; Labadie *et al.*, 2007; Scott, 2012; Giusti & Joaquim-Justo, 2013). Therefore, we focused solely on free E2 and its relation to gametogenesis. However, it remains largely unclear what determines the proportions of free to conjugated oestrogens in molluscs. Similar to the study of Peck *et al.* (2007) using the YES assay, no significant differences in E2 levels between males and females were obvious for the supplementary males subjected to the assay (results not shown). Thus, a sex-related difference in E2 as detected by Gauthier-Clerc *et al.* (2006) for *Mya arenaria* was considered to be rather unlikely. Moreover, our measurements using a competitive enzyme immunoassay confirmed the considerable variability in E2 levels encountered by Peck *et al.* (2007). Given the relatively low number of measurement points for free E2 levels in female gonads, the variability depicted in Figure 3 may simply reflect the variability in oocyte development within the population at a given sampling event. Overall, however, the pattern of E2 does not show any obvious relation to the gametogenic cycle. Notwithstanding the statistically significant differences that were obtained in spite of the high variability, high and low median values appear to be distributed rather randomly throughout the year.

When using oocyte volume as assessed by stereology, the negative linear correlation between E2 levels and oocyte volume fraction (Fig. 4) indicated that E2 levels may be higher during early oocyte development. Although this correlation is highly significant and Gauthier-Clerc *et al.* (2006) reported a similar relationship between oocyte maturation and E2 levels, the linear correlation coefficient (Pearson  $R = -0.53$ ) appears too weak as to be conclusive. In addition, only 28% of the variation in free E2 was explained by the  $\text{oV}_v$  ( $R^2 = 0.279$ ; Fig. 4), which means that both parameters appear to be unrelated. The absence of any relation between oogenesis and E2 in *D. polymorpha* in this study is consistent with the lack of a mechanistic model that could explain a role of E2 in gametogenesis, especially since molluscs do not synthesize oestrogen and lack the oestrogen receptors for steroid binding (Scott, 2012, 2013).

We can infer from the present data that although the general course of gametogenesis supposedly shows the same characteristics from year to year as indicated by the spawning in spring, this qualitative aspect can differ considerably in terms of oocyte volume and gonadal size in relation to the somatic tissue. This may be due to the lower temperatures in early spring 2006 as compared to 2005, which may have resulted in less food available for the animals, leading to reduced investment in gonadal growth. This illustrates the potential of quantitative procedures in the evaluation of gonadal development. However, which method will be most suitable to establish gametogenesis in bivalve molluscs ultimately depends on the specific research question being addressed.

## ACKNOWLEDGEMENTS

The authors greatly appreciated the assistance of the department of Pathological Anatomy, Jacques Monod Hospital at Le Havre, France. Thanks are also due to R. Triebkorn, Steinbeis-Transfer Center for Ecotoxicology and Ecophysiology, Rottenburg, Germany/Animal Physiological Ecology, Institute for Evolution and Ecology, University of Tübingen, Germany, for advice on stereological procedures and histological sections. The authors also express their gratitude to an anonymous reviewer and to J. Voltzow for their helpful comments on the manuscript. Special thanks go to all those who lent a hand during sampling, especially S. Olivier and E. Vince. This research was financed by the Interreg IIIA project RAED.

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