

Influence of diatoms on copepod reproduction. III. Consequences of abnormal oocyte maturation on reproductive factors in *Calanus helgolandicus*

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Abstract Observations of gonads and oocyte development stages (OS) have been achieved in *Calanus helgolandicus* females fed different algal diets and starved in filtered sea water under laboratory conditions during 8 days. The effects of 20 diets on egg production rates (EPR), hatching success (HS) and proportion of abnormal larvae (AL, development stages N1-2) were examined. With the control diet *Prorocentrum minimum* EPR and HS values were high, while AL was very low, coinciding with intact cell structures in oogonia (OO) and normal OS (OS1-OS4). With the other diets, oocyte maturation, EPR, HS and AL patterns were partially or totally impaired. Decrease of EPR coincided with the arrest of OS3 maturation and oocyte degradations, characterised by cell fragmentation, presence of apoptotic bodies in the OS3, degradation of cytoplasm in OS3 and OS4 and consequently the arrest of OS4 formation. These degradations were reversible when females were fed a favourable diet. Results reflect the presence of two distinct inhibitory mechanisms. Inhibition mechanism (1) impairs egg production. It was induced by starvation or by several species belonging to Bacillariophyceae (*Chaetoceros*

calcitrans, *Guinardia delicatula*, *Guinardia striata*, *Odonotella regia*, *Rhizosolenia setigera*, *Stephanopyxis turris*, *Thalassiosira pseudonana*) and mixed-diatom assemblages (collected in the field) and to the prymnesiophyte *Pavlova lutherii*. Remarkably other diatoms like *Navicula* sp., *Nitzschia* sp., *Skeletonema costatum* and *Thalassiosira rotula* did not induce mechanism (1) Inhibitory mechanism (2) affected exclusively HS and AL and was triggered by species independent of the production of polyunsaturated aldehydes (PUA), which are supposed to have adverse impacts on HS and larval development.

Introduction

Reproduction in calanoid copepods is influenced by temperature, food characteristics and sexual maturity of adults (Mauchline 1998; Ianora et al. 2003; Niehoff 2003, 2004; Arendt et al. 2005). Unlike higher crustaceans and insects, the type of hormones and the mechanism of hormonal regulation of gametogenesis, vitellogenesis, oocyte development, egg production and larval development are barely known in copepods (Adiyodi 1985; Blades-Eckelbarger 1986; Blades-Eckelbarger and Youngbluth 1984; DeLoof et al. 2001; Andersen et al. 2001; Pounds et al. 2002). Identification of gonad development stages is a significant indicator for spawning females (Niehoff and Hirche 1996). In copepods, information is scarce on the cytology and variability of oocytes during the vitellogenesis–oogenesis phases, which are paramount to the issue of spawning process (Niehoff and Hirch 1996; Mauchline 1998). Five oocyte development stages (OS) were identified during gonad maturation in *Calanus finmarchicus* by Niehoff and Hirch (1996). The tip of the ovary is occupied by oogonia (OO). The development of oocytes takes place

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in the prominent dorso-central gonad diverticulum and two lateral oviducts. During their maturation, oocytes change their shape and size, the morphology of the nucleus and the appearance of the ooplasm. Description of the four categories of OS (OS1–OS4) made by Niehoff and Hirsch (1996) applies to *C. helgolandicus*, as well. OO, OS1 and OS2 are visible in females belonging to copepodite development stage C5 and immature C6 females, whereas OS3 and OS4 are observed only in sexually mature females at stage C6. Development of the two OS1–OS2 stages occurs during the primary vitellogenesis, while the late OS3–OS4 maturation stages is achieved during the secondary vitellogenesis (Blades-Eckelbarger and Youngbluth 1984; Meuzy and Payen 1988). Links established by these authors between the oocyte development and vitellogenesis phases underline the key role played by maternal diets on the reproduction of calanoid copepods. In *Calanus* sp., females are fertilised once in their life, thus they do not request remating for spawning (Mauchline 1998). Consequently, during the breeding season key factors influencing egg production are mainly related to the characteristics of phytoplankton diets and gonad status of females.

Histological observation and detection of mature oocytes (OS4) have been proposed as a method for predicting egg production rates (EPR) during field survey (Runge 1987; Niehoff and Hirsch 1996; Niehoff 2004). Unfortunately, this method is open to several caveats related to the age of females and to diets in the field, generally unknown at time of sampling. While a link between EPR and female sexual maturity has been established in the laboratory under different food conditions (Razouls 1974; Razouls et al. 1987, 1991; Niehoff 2003; 2004), departure from normality ($\sim 30\text{--}40$ eggs female⁻¹ day⁻¹ in *C. helgolandicus*) is puzzling. Reduction of EPR down to 0–5 eggs female⁻¹ day⁻¹ can occur in pre-spawning or post-spawning C6 females, or when food is deficient or missing. Under these specific physiological conditions, the sequence of oocyte development OS2–OS4 is different and gonads normally regress (see Niehoff 1998). In these samples, it becomes difficult to identify the role of food factors, which are suspected to affect egg production (Paffenhöfer et al. 2005). The identification and selection of sexually mature females is crucial before running bioassays designed to evaluate the influence of food on copepod reproduction (Laabir et al. 1995; Lacoste et al. 2001). Poulet et al. (2006) have shown that variation in egg production in the field can be induced by ingestion of different diatom diets. Reversible-food tests with batches of females using a favourable control diet (PM) suggest that the females are not sterile but rather temporarily blocked by deleterious diets, or by starvation (Poulet et al. 1994, 2006; Lacoste et al. 2001; Ianora et al. 2004a).

This article compares the influence of different algal diets on the OS of *Calanus helgolandicus* by relating these physiological factors to the reproductive factors EPR, hatching success (HS) and abnormal larvae production (AL). It is the third contribution of a series of experiments on the variable reproductive patterns of calanoids in the coastal waters off Roscoff. The first contribution described the variations in time of the effects of diatoms in the field (Poulet et al. 2006). The second was focused on chemical factors in diets supposedly related to reproductive impairment of HS and AL (T. Wichard et al. 2006, submitted, Ph.D. Thesis; Wichard 2006). Herein, a description of variation of OS1–OS4 is used as an indicator of the maternal diet effects on reproduction.

Materials and methods

Sampling and selection of *Calanus* females

Calanus helgolandicus specimens were collected several times a week near Roscoff (48°45'N and 3°58'W, in the Western English Channel, France) during a spring–summer survey in 2003–2004, by towing a 500 μm mesh plankton net obliquely from 0 to 20 m. Samples were transported within 1–2 h to the laboratory, where adult, sexually mature females (12–30 in total, for each experiment) were sorted and incubated one individual per dish containing 100 ml of 0.22 μm filtered sea water during 24 h. These females were used to determine initial EPR, HS and AL values at day 1, reflecting their reproductive status under field conditions (Laabir et al. 1995).

Feeding experiments with single and mixed-species diets

Females used for testing the effect of single diatom diets on EPR, HS, AL and for the comparative histological studies, were kept in culture dishes containing *Thalassiosira rotula* (TR), *Rhizosolenia setigera* (RS), *Guinardia delicatula* (GD) or *Guinardia striata* (GS) set at a final cell density in the incubators corresponding to a mean concentration of 2.5×10^5 , 8.3×10^3 , 6.6×10^4 and 4×10^4 cells ml⁻¹, respectively. These mean cell densities corresponded to the concentrations of algae measured in triplicate in batch cultures, which varied between 20 and 35% of the mean during the entire feeding period. The corresponding carbon concentrations in the incubators were estimated for seven diets assayed for comparison of gonad histology (Table 1). The algal culture was renewed daily during the incubation period, which did not exceed 8 days. EPR, HS and AL were estimated daily following the techniques described by Laabir et al. (1995) and Poulet et al. (1995). Room temperature was set at $14 \pm 1^\circ\text{C}$ during all incubations. All the studied species occur in the Roscoff coastal waters during spring–summer blooms. Details concerning the isolation and cultivation of

Table 1 Summary of the reproductive responses (EPR, HS and AL) of *Calanus helgolandicus* females fed different diets

Group	Abbreviation	Species	Cell size (μm): length (l) or diameter (d)	Carbon content (pg/cell)	Food biomass in incubator ($\mu\text{g Carbon ml}^{-1}$)	PUA (5)	DHA/EPA (6)	EPR (7)	HS (6)	AL (6)
I	PM	<i>Prorocentrum minimum</i>	10 (l)	274 (1)	2.7	–	5.25	+	+	+
	TR	<i>Thalassiosira rotula</i>	40 (d)	157 (1)	7.9	+	0.28	+	+	+
II	CC	<i>Chaetoceros calcitrans</i>	4 (d)			–	n.d.	–	+	+
	GS	<i>Guinardia striata</i>	80–150 (l)	492 (2)	19.7	–	n.d.	–	+	+
	OR	<i>Odontella regia</i>	80–100 (l)			Traces	n.d.	–	+	+
	RS	<i>Rhizosolenia setigera</i>	250–300 (l)	500 (3)	4.2	–	0.16	–	+	+
	ST	<i>Stephanopyxis turris</i>	60 (l)			–	0.008	–	+	+
	NAV	<i>Navicula</i> sp.	14 (l)			–	0	+	–	–
III	NIT	<i>Nitzschia</i> sp.	20–30 (l)			–	0.07	+	–	–
	SK	<i>Skeletonema costatum</i> (RCC 75)	4–6 (l)			+	0.11	+	–	–
	TP	<i>Thalassiosira pseudonana</i> (CCMP 1335)	4 (d)			–	0.12	–	–	–
IV	GD	<i>Guinardia delicatula</i>	30–100 (l)	226 (5)	14.9	+	n.d.	–	–	–
	PL	<i>Pavlova lutherii</i>	3–4 (l)	160 (4)	16	–	n.d.	–	–	–
	NDA 1,3,5	Natural diatom assemblages	>11 (7)		<0.1–5	Variable in season	0.26–1.17	–	–	–
	NDA 1,2,5	Natural diatom assemblages				in season	0.26–1.17	–	–	–
	STAR	Filtered seawater						–	?	?

Abbreviations for the 20 diets assayed during the 2003–2004 survey are: CC (*Chaetoceros calcitrans*), GD (*Guinardia delicatula*), GS (*Guinardia striata*), NAV (*Navicula* sp.), natural diatom assemblages NDA (1: 13–20/05/03), (2: 17–25/07/03), (3: 28/03 to 01/04/04– used for histology observations), (4: 24–27/04/04), (5: 11–19/05/04), (6: 30/03 to 03/04/04), (7: 25/05 to 03/06/04), NIT (*Nitzschia* sp.), OR (*Odontella regia*), PL (*Pavlova lutherii*), PM (*Prorocentrum minimum*), SK (*Skeletonema costatum*), ST (*Stephanopyxis turris*), STAR (Starving condition in filtered sea water), TP (*Thalassiosira pseudonana*) and TR (*Thalassiosira rotula*). Diets in Groups I–IV categorised according to their specific effects on EPR, HS and AL: (+) favourable, (–) adverse effects. Estimates of carbon concentration of diets in incubators (PM, TR, RS GS, GD, PL, NDA1,3,5: used for histology observations) were based on carbon per cell data borrowed from (1) Ianora and Poulet (1993), (2) Dupuy et al. (2000), (3) Wiltshire and Dürselen (2004), (4) Verity et al. (1992), (5) Wichard et al. (2005) and (7) Poulet et al. (2006). Parallel measurements of both PUA and fatty acids [Wichard et al. 2005 (5); T. Wichard et al. 2006, submitted, Ph.D. Thesis (6)] were achieved during the feeding experiments [Poulet et al. 2006 (7)]. Details of EPR values, species, abundance and proportions of diatoms in the natural assemblages NDA1–7 (identified above by numbers and dates) and the chemical characteristics of diets (PUAs, DHA/EPA ratio) were given by Poulet et al. (2006) and T. Wichard et al. (2006, submitted, Ph.D. Thesis)

these diatom strains, as well as complete results on reproductive parameters (EPR, HS and AL), on food quality parameters (Chl α , C/N and polyunsaturated fatty acids), and on the content of polyunsaturated aldehydes (PUAs) are given by Poulet et al. (2006) and T. Wichard et al. (2006, submitted, Ph.D. Thesis).

Samples of natural diatom assemblages (NDA1–7), collected in 2003–2004 at the same station as copepod females, were used to test their effects on EPR, HS and AL (nauplius larva at the N1 and N2 stages). Among the seven mixed diets (NDA1–7; Table 1), only NDA1, 3 and 5 were tested for histology. To obtain these samples, 200 ml of sub-surface (1–2 m depth) sea water samples were gently filtered by gravity through a filtering-column formed of two Sartorius filtering funnels, the top one supporting a 350 μm mesh and the one below a 11 μm mesh Nitex sieve (Millipore Tokyo, Japan, 45 mm diameter). The top one was used to remove zooplankton and large particles, while the second

was used to collect diatoms. Samples were collected on the 11 μm mesh and re-suspended in incubators containing 100 ml filtered sea water (Millipore, 0.22 μm). The final concentration of NDA diet in each incubator was approximately two times higher than the initial concentration in nature. Details for this procedure, identification, abundance of diatom species in NDA, sampling dates (see legend Table 1) and are described by Poulet et al. (2006).

The dinoflagellate *Prorocentrum minimum* (PM) was used as a control diet at concentrations corresponding to 10^3 – 10^4 cells ml^{-1} in the incubators. The growth conditions of this alga have been described earlier (Poulet et al. 1994). This non-diatom diet was used to test the reproductive capacity of *C. helgolandicus*, thus verifying if low EPR were due to the fact that females needed to remate, or were at the pre- or post-spawning stages. The prymnesiophyte *Pavlova lutherii* (PL) was used as a second non-diatom control, at concentrations corresponding to 10^5 cells ml^{-1}

in the incubators. The growth conditions of this alga have been described earlier (Lacoste et al. 2001).

Experiments with starved females

In a group of carefully selected females, 30 spawning specimens, identified at the end of a preliminary 24 h incubation period, were individually placed in incubators containing 100 ml of filtered sea water (Millipore, 0.22 μm). The filtered sea water was renewed daily during the 8-day incubation period. Four starved females were sacrificed and fixed at day 1 (reflecting initial diet condition in the field) and the following days 2, 4 and 8 (reflecting the increasing starvation). EPR, HS and AL were recorded daily until the end of incubation period with the remaining females.

Sampling of females for histology

Batch samples, 4–5 females each, used for the histology observation of gonads were randomly collected at the end of each incubation series, at days 4–8, depending on the diets (GD, GS, TR, RS, NDA1-3-5 and PM: see details in Poulet et al. 2006). At the end of the 5–8 days incubation periods with PM, TR and NDA necessary to evaluate EPR, HS and AL influenced by each diet, females were incubated another 24 h with the same diet and four batches of four females each were sampled and fixed at four different times T1–T4, with about 6 h interval between each sampling time (see Figs. 5, 6, 7). This procedure allowed observation of the circadian variation of oocyte development phases. Egg production in *C. helgolandicus* is characterised by a daily cycle, with EP occurring generally once between 03 and 08 h in the morning (Laabir et al. 1995). Because the timing between each egg-clutch is neither synchronised nor predictable among females, we applied this procedure to detect all types of oocytes, including late oocytes OS3 and OS4, before spawning. With starved females, the same procedure was applied to measure daily EPR, HS, AL and morphology of oocytes at days 4 and 8. The aim was to compare the status of oocyte maturation in gonads between starved females and females fed with favourable or deleterious diets.

At three different occasions (26/06/03, 07/07/03 and 29/03/04), batches of 4–6 females presenting exceptionally low EPR values (0–5 eggs female⁻¹ day⁻¹) at the end of the 24 h pre-incubation in filtered sea water were fixed for histological examination of gonads (see Fig. 8, in situ). These females are assumed to reflect unsatisfactory field-diet conditions at time of sampling.

Histology preparation and observation of gonads

After 1, 4 and 6–8 days depending on the type of test before, or after egg laying, females were sacrificed and fixed for

microscopic examination of gonads and oocytes. Females were fixed for 48 h in 1% paraformaldehyde and 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2). Specimens were dehydrated using standard ethanol (RPE, Carlo Erba) series. Impregnation was performed using Spurr (EMS) series and final embedding was made with Spurr and left to polymerise for 12 h at 70°C. Semi-thin sections (2–3 μm) were cut with glass knives on a LKB microtome and stained with toluidine blue for 30 s prior to examination under an Olympus microscope (Olympus BX61). Samples of semi-thin sections were prepared following transversal sections Z1–Z6 and longitudinal sections Z7 in two females or more per sample (Fig. 3a). Comparisons between gonads were based on sections corresponding to the same areas shown in Fig. 3. Photographs were taken at the same magnification (200 \times) using a digital Spot RT cooled CCD camera and further computerised using the Spot basic program.

Results

A summary of the favourable and deleterious effects of diets on three reproductive factors in *C. helgolandicus* females is reported in Table 1. The reproductive responses were rather fast and varied significantly after 2–3 days (see details in Poulet et al. 2006; T. Wichard et al. 2006, submitted, Ph.D. Thesis). Complementary results in Fig. 1 show that significant variations of EPR, HS and AL were observed with females fed PL or starved in filtered sea water (Wilcoxon non-parametric rank sum test, $P < 0.001$). When EPR values decreased with GD, GS, OR, RS and NDA diets, we verified if the reproductive inhibition was truly induced by such diets. In the example shown in Fig. 2, EPR, HS and AL values at day 1 corresponded to initial field diet condition. The same females were further fed PM and NDA diets, successively. Similar results as shown in Fig. 2 were achieved with NDA5 (not shown). EPR and HS values returned to normal at days 4–7. Beyond day 7, HS decreased to zero, whereas AL increased drastically. This reversibility further supports that variation of the three reproductive factors observed before and after PM diet shift were not due to female age, but rather to the maternal-food effect.

A functional link has been established between variation of EPR values, morphology of gonads and OS in different batches of females fed PM, TR, NDA3, GD, GS, RS, PL and with starved females fixed at the end of the 4–8-day incubation periods (see arrows in Fig. 1). These bioassays were achieved with different batches of females at several periods in 2004 (see details in Table 1; Poulet et al. 2006). Position of gonad and oviduct, distribution of oocytes in a fertile *Calanus* female are shown in Fig. 3a, which also indicates the relative position and types of semi-thin sections made in the samples, following transversal (Z1-6),

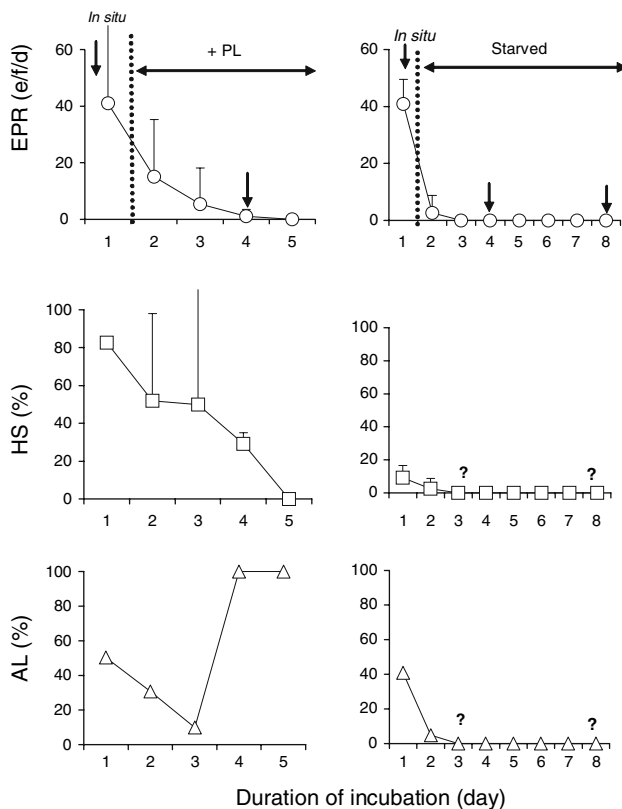


Fig. 1 Variation in egg production rates (*EPR*), hatching success (*HS*) and proportion of abnormal larvae (*AL*) in different batches of females fed one non-diatom diet (*PL*) and starved in filtered sea water (*STAR* in Table 1). Day 1 (*left arrow*): estimates of reproductive responses in the field before start of bioassays. *Horizontal arrows* indicate duration of feeding incubation with the same food. *Arrows (bottom and right)* indicate sampling day of females fixed for histological observations. Values are mean and standard deviation (*bars*). (?): no *HS* and *AL* values beyond day 4 because of arrest of EP

longitudinal and/or sagittal (*Z7*) axes. To allow reliable comparisons between maternal-food effects on gonads, light-microscope observations and photos were taken for the same sections *Z1–7* for all female samples (Figs. 3b, 4, 7, 8, 9, 10).

Photos in Fig. 3b show two series of semi-thin sections taken along similar transversal *Z1–Z6* axes, allowing visualisation of gonad anomalies induced by the ingestion of *NDA1* diet in comparison to control *PM*. Females used in these examples were randomly collected in the morning after spawning time and thus, *OS4* stage was not visible in female fed *PM*. With *PM*, all oocyte stages *OO* to late *OS3* showed a normal aspect and were perfectly distributed along the gonad diverticulum and the oviduct canal. With *NDA1*, normal *OO*, *OS1*, *OS2* and early *OS3* were observed in the gonad, whereas the late *OS3* development stages were always missing. Moreover, the entire gonad had shrunken, due to the missing oldest *OS3* stages in the *Z1–Z4* sections and in oviducts (Fig. 3b).

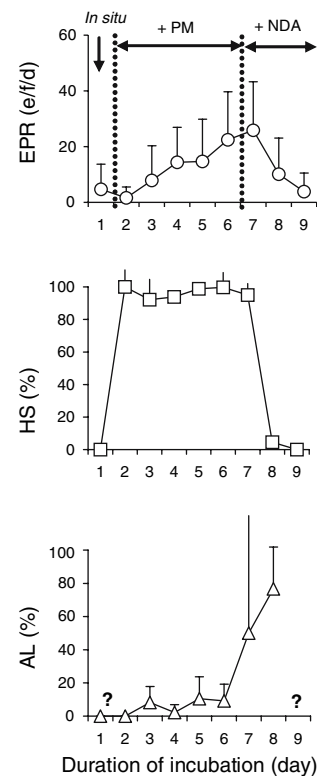
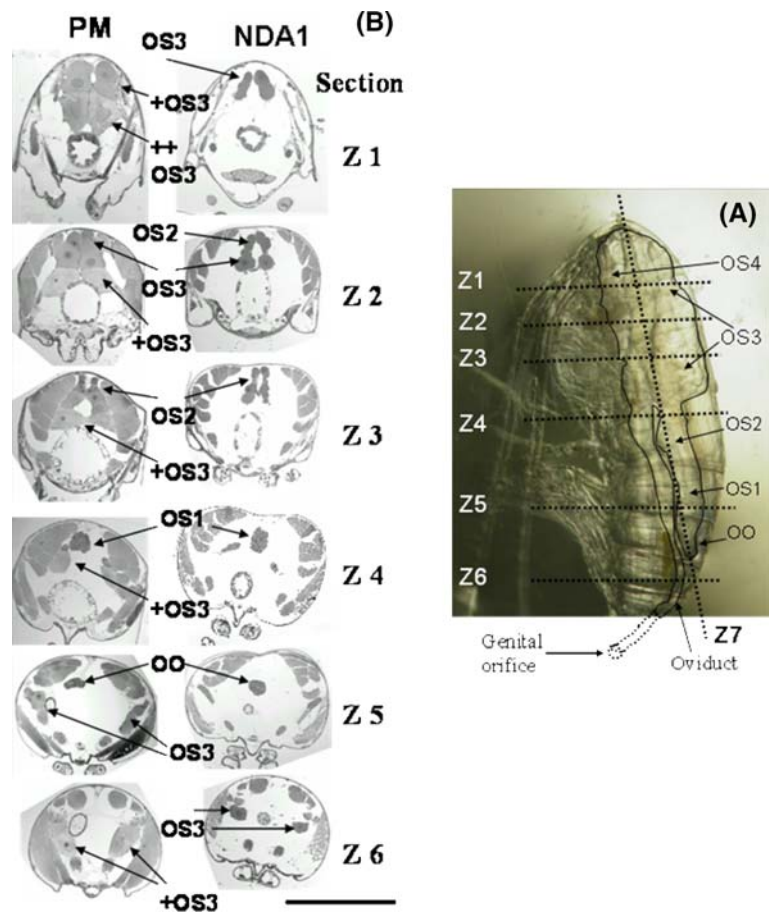


Fig. 2 Effects of diet on the reproductive responses (*EPR*, *HS* and *AL*) of females fed in the field (*in situ*; day 1) and shifted to favourable (*PM*) and unfavourable (*NDA3*) diets. Values are mean and standard deviation (*bars*). (?): no *AL* values at days 1 and 9 because of arrest of hatching

Two series of photos illustrate the aspects of each oocyte development stage, *OO* to *OS4*, in gonads of females fed single *PM* and *TR* diets (Fig. 4 left: normal maturation) and fed the deleterious diets *GD*, *GS*, *RS*, *NDA3* or *PL*, as well as starved (Fig. 4 right: abnormal maturation). With *PM* and *TR*, rows of oogonia *OO*, oocytes *OS1*, *OS2*, early and advanced *OS3* and *OS4* presented regular shape, normal dense homogeneous nucleus and nucleolus, with presence of normal dark staining mass in *OS2* and a progressive, regular vacuolisation in the advanced *OS3* and *OS4* development stages. In general, no gaps between cells were observed. In the late *OS3*, normal maturation was followed by disappearance of the nucleus membrane, corresponding to the germinal vesicle breakdown (arrow: *GVBD*). In the *OS4*, chromosomes had migrated to the equatorial plate of the metaphase *M1* (arrow) few hours before spawning. Numerous, distinct follicle cells were visible at the border of the *OS3* and *OS4* cells. With *GD*, *GS*, *RS* and *NDA3* diets, cell structures observed in the *OO*, *OS1* and *OS2* stages appeared morphologically normal (Fig. 4 left). Gaps between cells were scarce in the *OS2* layers, but they occurred more frequently when food concentration was high, or when incubations had lasted longer than 8 days with a specific deleterious diet (not shown). When gonads

Fig. 3 *Calanus helgolandicus*. **a** Photograph of lateral, left view of a sexually mature female. Envelope shows the space occupied by gonad and oviduct analysed during the study. Histological and cytological observations of gonads and oocytes compared between semi-thin transversal sections Z1–Z6 and longitudinal and/or sagittal sections Z7. Relative oocyte positions in gonad are given by arrows- OO oogonia, OS1–OS4 oocyte development stages observed in spawning females. **b** Photos of semi-thin transversal sections Z1–Z6 in females fed two different diets. Comparison between morphology of gonads and different oocyte categories (arrows), reflecting the favourable (PM) and deleterious (NDA1) effects of maternal diets on oocyte maturation. OS3: early oocyte. +OS3: advanced oocyte. ++OS3: late oocyte. Scale bar: 400 μ m

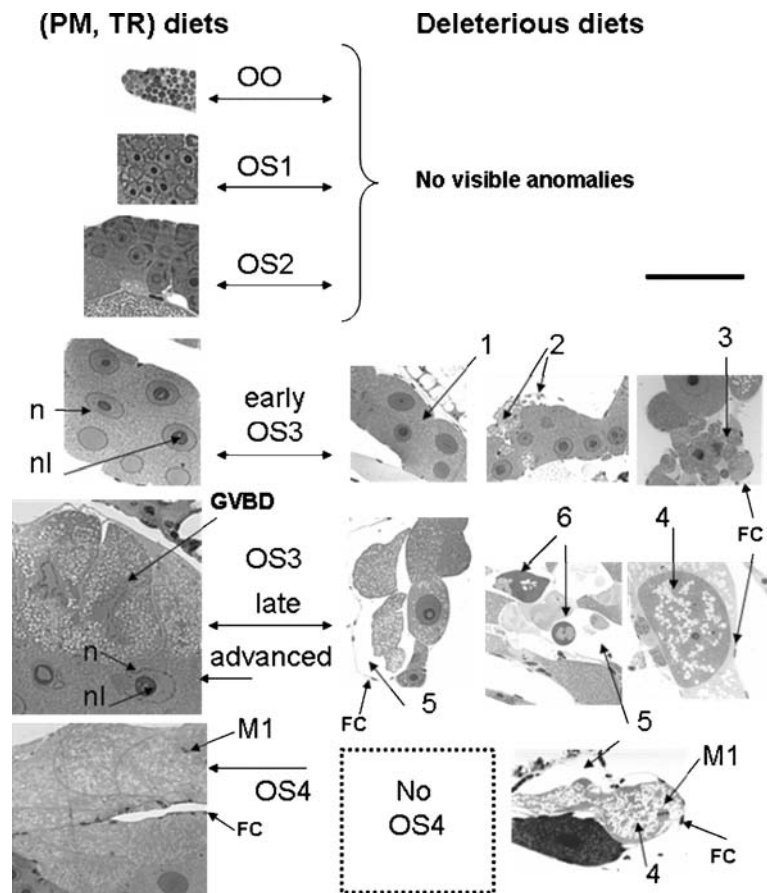


had reached such level of degradation in OS2, recovery of egg production was either very poor, or took longer during the reversible PM test. With deleterious diets, or starving condition, samples presented many oocytes blocked at the early OS3 stage, which appeared morphologically normal (Fig. 4: 1, 2). OS3 showed several cell fragmentations and apoptotic bodies (Fig. 4: 3, 6). Gaps due to mild or heavy cytoplasm degradations became more frequent in the early, late and advanced OS3 oocyte stages (Fig. 4: 2, 4, 5). Intense degradation in the ooplasm occurred, whereas empty spaces in the cytoplasm of late oocytes appeared like empty or shrunken gonads. Follicles seemed normal. The other type of anomaly was related to heavy cell degradation in late OS3 (Fig. 4: 5). OS4 were also highly degraded or had disappeared, in conjunction with very low values, or decreased EPR values (Fig. 1; Poulet et al. 2006). Formation of late OS3 stage was rather fast (<6 h). This oocyte category was thus barely observed even in normal, non-inhibited females (Fig. 4, left).

In most female samples collected in the morning (8.30–9.30 hours: Figs. 5, 6, 7, 8, 9, 10) after egg laying, advanced or late OS3 and OS4 oocytes were often missing, due to sampling time. In order to improve the probability to observe late oocytes, we had to match the sampling time

with female circadian spawning cycle. With PM, TR and NDA3 diets, samples were randomly collected at four different times, in order to detect major phases of the oocyte maturation (Figs. 5, 6, 7). Photos of longitudinal sections Z7 are given for two favourable (PM: Fig. 5 and TR: Fig. 6) and one deleterious (NDA3: Fig. 7) diets for only one individual among four replicate females per batch samples, which was examined at each sampling time. They illustrate morphological variations occurring in the gonads during a day–night cycle. With PM, the gonads were fully developed and occupied the entire diverticula. OO at the tip of the ovaries, and the four types of normal oocytes OS1–OS4 were observed in this series. With this sampling procedure the oldest stages (advanced or late OS3 and OS4) could be observed at time T3 and T4. They presented similar and normal cell structures, as described earlier in *C. finmarchicus* by Niehoff and Hirsch (1996), Niehoff (2004). The morphology of gonads slightly changed with time. At T1, corresponding to the post-spawning phase OS1, OS2 and early OS3 were clearly identified in the entire diverticula. At times T2 and T3 corresponding to the spawning interphase, oocytes were abundant, with late OS3 started to mature. At T4 corresponding to pre-spawning phase, numerous late OS3 and new OS4 were detected (Fig. 5).

Fig. 4 Light-microscope photos of different oocyte development stages observed in gonads of females fed diets affecting oocyte maturation and EPR values. *Left*: The positive effect of PM and TR was reflected by series of normal oocyte OS1–OS4. *Right*: The adverse effect of GD, GS, RS, NDA3, PL and starvation on EPR was generally not visible at the OO, OS1, OS2 oocyte stages. Cell anomalies 1–6 (arrows) observed in oldest oocyte stages OS3 and OS4. About 1- arrest of OS maturation without visible cell degradation, 2- weak degradation of cytoplasm, 3- cell fragmentation, 4- abnormal granules in cytoplasm, 5- deep cytoplasm degradation leading to empty ooplasm, and 6- apoptotic bodies. *M1* chromosomes at the equatorial metaphase plate. *n* nucleus, *nl* nucleolus, *FC* follicular cells. Magnification: $\times 200$. Scale bar: 400 μm



With TR, the gonad morphology and the status of OO and oocytes in the diverticulum were similar to PM, apparently undergoing normal maturation between early OS3 and OS4 at times T1–T4 (Fig. 6). With NDA3 diet, the morphology of gonads was deeply modified, mainly in the frontal and thoracic regions (Fig. 7). Cell damage was observed at times T1–T4 during the entire circadian cycle, mainly in the early and advanced OS3 stages. These anomalies resembled those shown in Fig. 4. Based on the low number or absence of late OS3 in samples collected at times T1–T4 (Fig. 7), we infer that the NDA3 diet also arrested maturation of OS3. Few OS3 had matured into OS4 (only 1/4 of examined females; see T4, Fig. 7). This observation was supported by very low EPR values measured at day 5 in the remaining females (days 8–9 in Fig. 2, see detail values for other NDA diets in Poulet et al. 2006).

The cytological impact of other diatom diets RS, GD and GS on the gonad morphology reveals cell degradations in gonads of females collected at three different periods (26/06/03, 07/07/03 and 29/03/04; Fig. 8). When *C. helgolandicus* had fed on natural phytoplankton samples (i.e. NDA + non-diatom food), they were very low spawners ($\text{EPR} < 5 \text{ eggs female}^{-1} \text{ day}^{-1}$). During these bioassays, females were sampled, at the end of the incubation period around 9 h, corresponding to the early post-spawning

phase. A strong inhibitory effect was observed in the oocyte stages of the females at day 1 (Fig. 8: in situ), following 24 h incubation in filtered sea water. With RS, maturation of early OS3 into advanced OS3 was arrested at day 4. Thus, advanced OS3 were completely missing and production of OS4 was impossible. As a consequence, the entire frontal and thoracic areas in the ovaries were very slim and resembled gonads of immature females (see Niehoff 1998). However, results achieved with the PM-reversible assays following several days of incubation with RS and NDA3 demonstrated that females could recover their potency, suggesting that EPR was temporarily blocked by deleterious diets (Fig. 2; Poulet et al. 2006; T. Wichard et al. 2006, submitted, Ph.D. Thesis). The gonad degradation observed in females fed with GD and GS was less than with RS. At days 6–7, OS4 had disappeared in the ovaries of the majority of females fed GD and GS, as confirmed by very low EPR values (see results in Poulet et al. 2006). Advanced OS3 were very scarce and/or showed cells with highly degraded ooplasm, reflecting abnormal OS3 maturation or arrested OS3 maturation in the majority of females (Fig. 8). Samples for histology were not collected with OR diet nor with other diets tested during the 2003–2004 survey reported in Table 1.

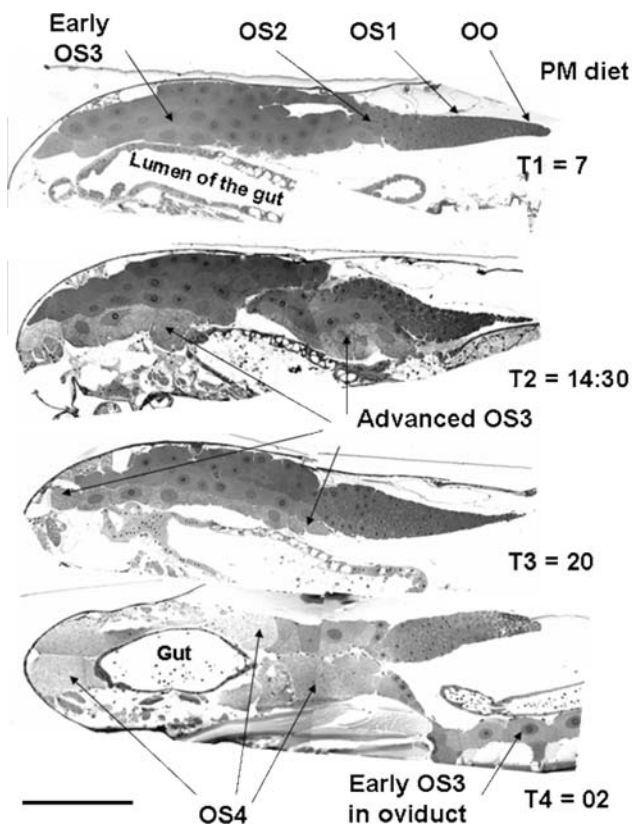


Fig. 5 PM diet and circadian variation of oocyte maturation in gonads. Photos of semi-thin longitudinal sections Z7 made in females fixed at times T1, T2, T3 and T4 (in hour). Spawning had occurred before T1, explaining absence of OS4 at T1 and T2. Rows of oogonia OO and other oocytes OS1–OS4 were normally distributed in the gonad. Magnification: $\times 200$. Scale bar: 400 μm

Comparison of the maternal-food effects between females fed RS, NDA3, PL and starved was achieved at day 4 during successive incubation tests (Fig. 1; Poulet et al. 2006). Examination of samples along a similar longitudinal section Z7 suggested that gonad degradation was different among these four diets. The degree of degradation and frequency of cell anomalies in gonads was most pronounced in RS followed by NDA3 and PL. With these diets, degradation was deeper than in starved females at day 4 (Fig. 9). At day 8, gonad degradation in starved females was equivalent or deeper than these induced by the other deleterious GD, GS diets at days 6–7 (Figs. 7, 9). Gonad degradation in the selected in situ females at day 1 (Fig. 8) was as high as in females fed NDA3 or starved at day 4 (Fig. 9). This type of gonad degradation was reversible when starved, or non-starved females were shifted to PM diet (gonads returning to normal morphology in 3–4 days; see Fig. 2; Lacoste et al. 2001).

In general, oocytes in oviducts could not be observed in the Z7 sections shown in Figs. 5, 6, 7, 8, 9, because of the lateral position of oviducts in the body. Additional sagittal Z7 sections made in the same female samples as described

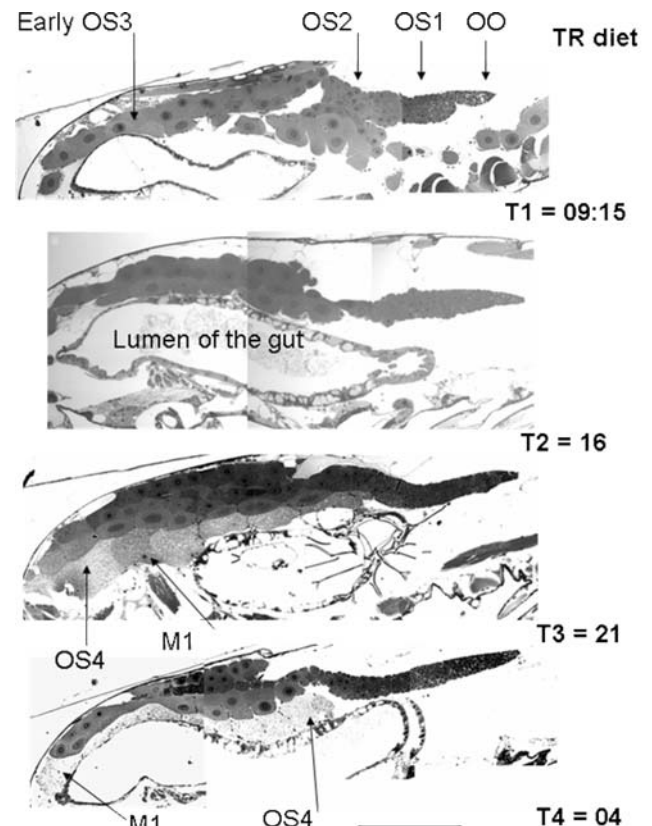


Fig. 6 TR diet and circadian variation of oocytes maturation in gonads. Photos of semi-thin longitudinal sections Z7 made in females fixed at times T1, T2, T3 and T4 (in hour). Rows of normal OS4 were visible in the gonad at times T3–T4, while spawning had occurred before T1, explaining absence of OS4 at T1 and T2. Rows of oogonia OO and other oocytes OS1–OS4 were normally distributed in the gonad. Magnification: $\times 200$. Scale bar: 400 μm

above, or in spare samples permitted to visualise variations of oocyte maturation in oviducts (Fig. 10). Cell degradation symptoms were the same as described above (Fig. 4). These complementary results reveal that several food regimes can impair maturation of OS3 oocyte stages and induce cell degradation in OS3–OS4 in the entire reproductive system of *C. helgolandicus* females (Figs. 3, 4, 5, 6, 7, 8, 9, 10).

Discussion

Our results on the maternal-food effects on the reproduction of *C. helgolandicus* revealed a close link between morphology of gonads, oocyte maturation and variation of EPR. HS or AL impairments were not related systematically to the egg production performance (Table 1; Figs. 1, 2, 4, 5, 6, 7, 8, 9, 10). The first functional symptom caused by unfavourable diets was the inhibition of oocyte maturation followed by degradation and decrease in egg production (Figs. 1, 2, 3b, 4). These results are consistent with previous reports

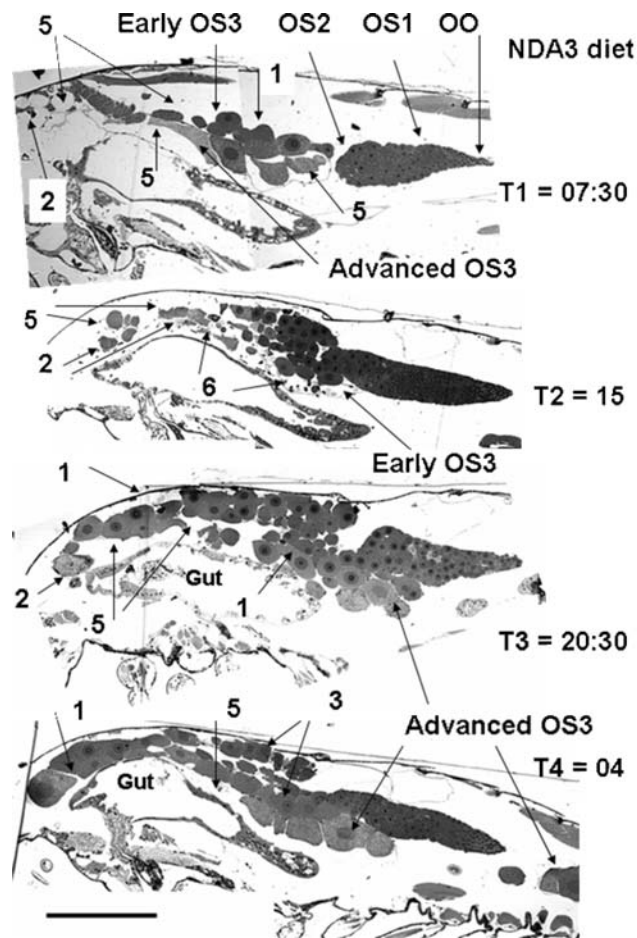


Fig. 7 Deleterious effect of NDA3 diet and circadian variation of oocyte maturation in gonads. Females were fixed at times T1, T2, T3 and T4 (in hour). Normal oogonia OO and OS2 oocyte stages were regularly and normally distributed in several rows. Abnormal cell symptoms were characterised by arrest of OS3 maturation (1: as in Fig. 4), different cell degradations in early and advanced OS3 stages (2, 3, 6: as in Fig. 4), large empty spaces in ooplasm (5: as in Fig. 4). Absence of late OS3 and OS4 was frequent (see time T3 and T4). Magnification: $\times 200$. Scale bar: 400 μm

(Razouls 1974; Razouls et al. 1987, 1991; Niehoff 1998, 2003, 2004; Niehoff and Hirsch 1996; Lacoste et al. 2001; Niehoff et al. 2002). Cell symptoms in *C. helgolandicus* oocytes were identified (Fig. 4) and related to: (1) arrest of OS3 maturation, (2) formation of cell fragmentations, (3) formation of apoptotic bodies (Kerr et al. 1972; Wyllie et al. 1980; Nezis et al. 2003), (4) degradation of cytoplasm and (5) decrease, or arrest of OS4 formation. Anomalies were triggered either by certain diatom species, by a prymnesiophyte or by mixed diatom assemblages. Not all diatom diets had the same effect on EPR, HS and AL, simultaneously (Table 1; Figs. 1, 2, 4, 5, 6, 7, 8, 9, 10). TR was favourable to EPR. However, a slight inhibition of HS and negative effect on larval development occurred at day ≥ 7 . (T. Wichard et al. 2006, submitted, Ph.D. Thesis). The activity thresholds of TR, or SK on HS is supposedly

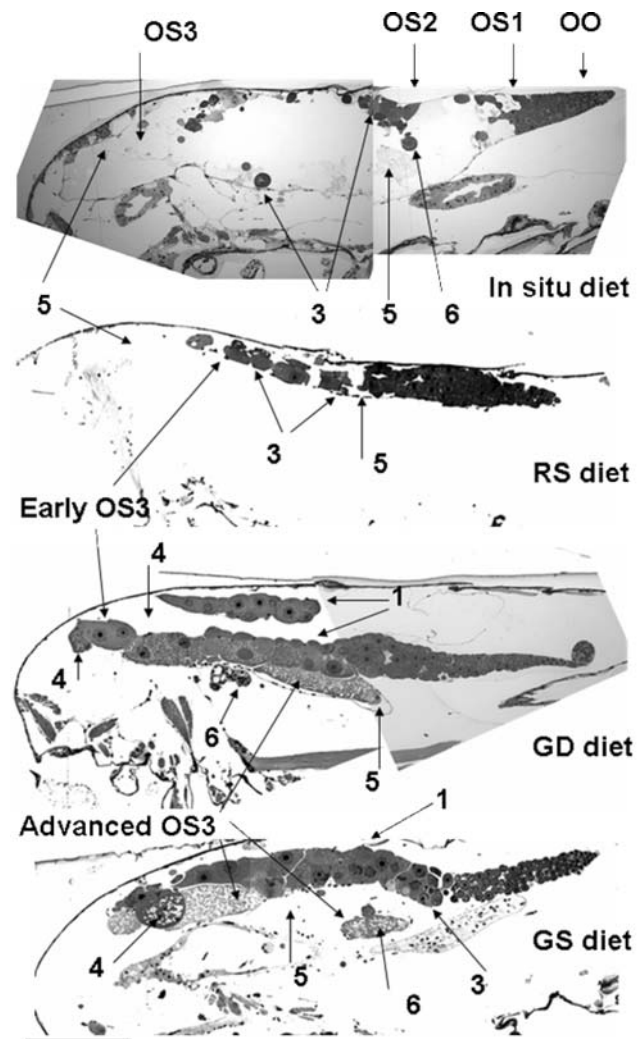


Fig. 8 Deleterious effects of RS (at day 4), GD (at day 6) and GS (at day 7) diets on gonad morphology and oocyte development stages in females fixed at time T (8.00 hours $< T < 9.30$ hours). In situ diet: gonad anomalies in females fed natural phytoplankton mixed diets in the field (non-diatom + NDA diets) and presenting low EPR values ($0\text{--}5$ eggs female $^{-1}$ day $^{-1}$) at incubation day 1 in filtered seawater. Oogonia OO and OS1–OS2 oocyte development stages looked normal in diets, except with RS and in situ diet. Cell symptoms were characterised by the arrest of OS3 maturation (1), cell fragmentation in OS3 stages (3), abnormal granules in cytoplasm (4), complete elimination of late OS3 and OS4 (5), living large empty spaces within the ovaries or resulting in the shrinkage of gonad, and apoptotic bodies (6). Magnification: $\times 200$. Scale bar: 400 μm

diatom-strain dependent (Pohnert et al. 2002; Ask et al. 2006). Obviously the TR species isolated during this study behaves differently compared to previously investigated TR strains (Pohnert et al. 2002; Ianora et al. 2003). GD, GS, OR, RS and mixed diatom species in NDA1–7 diets were all detrimental to EPR. In contrast, CC, GS, OR, RS and ST had a favourable effect on HS and AL, whereas no positive effects on the reproductive response were observed with NAV, NIT, GD, TP (Table 1).

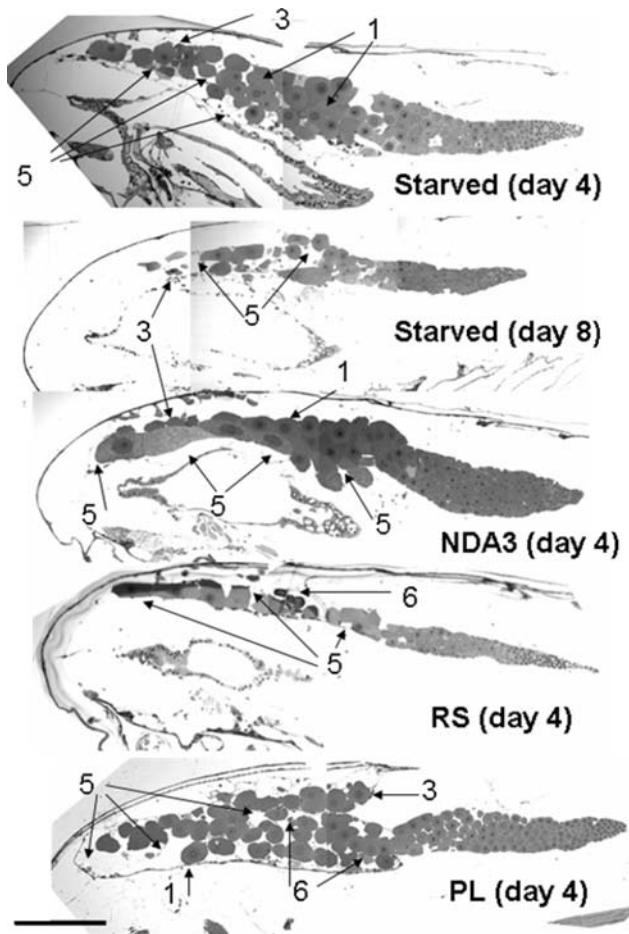


Fig. 9 Comparison of gonad morphology and oocyte degradations in females starved in filtered sea water fixed at days 4 and 8, and females fed NDA3, RS and PL, fixed at day 4. Abnormal cell symptoms were characterised by arrest of OS3 maturation (1), cell degradation in early and advanced OS3 stages (3, 6), large empty spaces in ooplasm (5). Late OS3 and OS4 were rarely observed. Magnification: $\times 200$. Scale bar: 400 μm

Oocyte degradation was triggered either by GD (a weak PUA producer), GS or RS (two non-PUA producers; Wichard et al. 2005). PL (a non-PUA producer; T. Wichard, unpublished) had a deleterious effect as well (Fig. 1). OR also known as a weak-PUA producer arrested EPR (Wichard et al. 2005, Table 1; Poulet et al. 2006). In contrast, TR, one of the strongest PUA producers, neither altered gonad morphology nor EPR. SK, another PUA producer, was favourable to EPR as well (Wichard et al. 2005, Table 1; Ask et al. 2006). Indeed, these results support the idea that oocyte anomalies and decrease of EPR were not related to PUA production (see also Poulet et al. 2006) and are not restricted to diatoms. Three species belonging to prymnesiophytes (*P. lutherii*, Fig. 3; Lacoste et al. 2001), chlorophytes (*Dunaliella tertiolecta*, Lacoste et al. 2001) and dinoflagellates (*Alexandrium tamarense*, Ianora et al. 2004b) could also block egg production. Accordingly, at

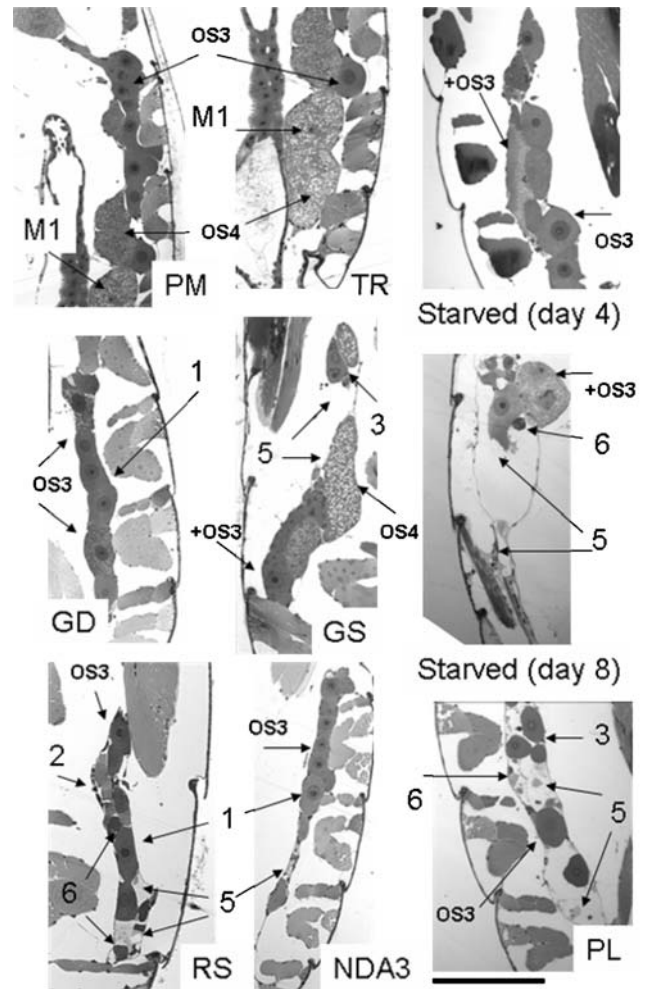


Fig. 10 Light-microscope photos of oviducts (left or right views of sagittal Z7 sections) from females fed different diets (PM, TR, GD, GS, RS, PL, NDA3, or starved) and fixed on different dates at days 4, 7 and 8 in the morning at time T (8.00 h < T < 9.30 h). Types of oocytes (OS3, +OS3 and OS4) and categories of cell symptoms (1, 2, 3, 5, 6) were same as in Figs. 3b–9. Scale bar: 400 μm

least one inhibitory mechanism (1: Fig. 11; Table 1) specifically targeting OS3 maturation and EPR inhibition is expressed by phytoplankton species belonging to different families. Results further imply that a second mechanism (2: Fig. 11) is involved, which affects exclusively HS or/and AL (NAV, NIT and SK: Table 1; see also Halsband-Lenk et al. 2005; Vargas et al. 2006). Only few phytoplankton species seem to possess simultaneously mechanisms (1) and (2) (GD, PL and TP: Table 1).

The deleterious effect of phytoplankton diets on the oocyte maturation may be caused by several factors. First, unidentified algal toxins involved in mechanism (1) could target OS3 maturation (Fig. 11). Second, PUAs and/or closely related toxic metabolites could influence directly or indirectly mechanism (2) (Fig. 11), as suggested earlier (Miralto et al. 1999; Ianora et al. 2004a; see review by

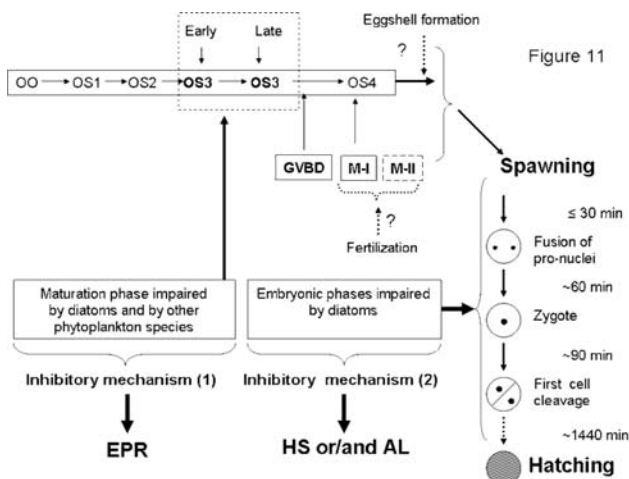


Fig. 11 Diagram summarizing the different phases of oocyte maturation in gonad before spawning and key cell events observed in embryo before hatching. OO oogenesis. OS1–4 oocytes development stages 1–4. GVBD germinal vesicle breakdown. Timing of fertilisation occurring at the M-I, M-II transition and eggshell formation (see Cuoc et al. 1994; Barthelemy et al. 2001) are not known in *Calanus helgolandicus* females and thus, the influence of diets during these phases is ignored. Normal oocyte maturation OS3–OS4 leads to normal egg production rates (EPR), hatching success (HS) and larval development. Inhibitory mechanism (1) induces cell anomalies observed during OS3 maturation (Figs. 3b, 4, 5, 6, 7, 8, 9, 10) and decrease or inhibition of spawning (EPR: see Figs. 1, 2, Table 1 and detail values in Poulet et al. 2006). Inhibitory mechanism (2) impairs hatching success (HS), ultimately hatched embryos lead to abnormal larval (AL). It does not affect EPR (see Figs. 3b, 4, 5, 6, 7, 8, 9, 10; Table 1). Embryonic phases have been described by Poulet et al. (1994, 1995, 2003), Buttino et al. (2004) and Ianora et al. (2004a)

Pohnert 2005). Third, essential precursors, or specific essential nutrients involved in vitellogenesis 2 could be deficient or lacking, thus disturbing successively the oocyte and embryonic maturation processes (see e.g. Lee et al. 1999; Andrade and Lee 2001; cholesterol: Hasset 2004; DHA: Arendt et al. 2005).

PUAs are believed to be harmful to copepod reproduction (for reviews: see Ianora et al. 2003; Paffenhöfer et al. 2005; Pohnert 2005). In our study, histological changes were however unrelated to the levels of PUAs in diets (Table 1). The role of diatom-rich diets for copepod reproduction is often questioned on the grounds of nutritional adequacy and toxicity (see review by Paffenhöfer et al. 2005). Based on our simple experiments, it is difficult to determine whether the maturation and reproductive factors (EPR, HS and AL) were impaired by diatom toxic metabolites rather than by food limitation (Table 1). Nevertheless, the fact that none of the mixed diatom diets NDA1–7 (Table 1) had positive effect on any of the investigated reproductive factors may indicate the presence of a toxin.

Oocyte maturation is strongly linked to vitellogenesis, a highly energy demanding process, which is especially affected by limited supply of nutrients. Our experimental

design rules out a food-deficiency with diatom concentrations in assays increased by factor of 2–4 in term of carbon content compared to the field situation. Thus nutrient deficiency seems not to be the exclusive limiting factor. Comparable gonad degradation and cascade reproduction-inhibitory processes have been described as well in other invertebrates and were attributed to specific inhibitors (Matova and Cooley 2001; Tsukimura 2001). Earlier studies showed that in calanoid copepods, the number of OS OS3–OS4 and EPR increase are related to the rise of food concentration (Niehoff 2003). With the NDA1–7 tests however, a reverse pattern was found in relation to food concentration increase (Fig. 2; Poulet et al. 2006). According to Delaunay et al. (1993), Berge et al. (1995) and Lacoste et al. (2001), SK, TR and NDA diets and the non-diatom PL do not lack essential polyunsaturated fatty acids. The fact that the cell degradation in gonads of starved females resembled those of specimens fed PL, RS, GD, GS and NDA3 at day 8 is also puzzling, because these effects could be attributed to nutrient deficiency or to reduced metabolism at starvation. At days 1 and 4, however the latter is unlikely, because oocyte damage induced by GD, GS, PL, RS, NDA3 and in females fed natural phytoplankton-mixed diets (non-diatoms + NDA: in situ, Fig. 8) was higher than in starved females (Figs. 9, 10). As reported earlier by Lacoste et al. (2001) with *Calanus helgolandicus* and by Niehoff (2004) with *C. finmarchicus*, proportion of spawning females was reduced and clutch size remained low during starvation (<5 eggs female⁻¹ day⁻¹). Niehoff (2004) reported that when food was limited or during starvation, gonads appeared smaller while low spawning activity was maintained. In contrast, oocyte disintegration was observed, as shown in Fig. 9, suggesting that vitellogenesis was fuelled by recycling internal reserves. Nevertheless, results also show that while food in assays was always higher than food concentration in the field at day 1 (Poulet et al. 2006), the normal oocyte maturation and egg production processes were promptly or progressively shunted the following days (depending on diets). Several authors have identified various chemicals (EPA, DHA, 18:4 ω 3, 18:3 ω 3, PUFA and cholesterol) considered as essential compounds for crustacean reproduction (Jónasdóttir 1994; Lee et al. 1999; Andrade and Lee 2001; Hasset 2004; Maps et al. 2005; Arendt et al. 2005). Diatoms might be not an adequate food supply, because they harbour less DHA and thus have a low DHA/EPA ratio. According to Arendt et al. (2005), a beneficial ratio is in the range from 2.5 to 4.8. In Table 1, reported DHA/EPA values were <1.2, except for PM. However, in the Roscoff coastal waters, *C. helgolandicus* reproductive responses were not correlated to any chemical factors, such as polyunsaturated fatty acids or PUAs, estimated in the diets (see Table 1; T. Wichard et al. 2006, submitted, Ph.D. Thesis).

Investigations on the role of fatty acids on copepod reproduction have generated contradictory results. This implies that polyunsaturated fatty acids (PUFA, EPA and DHA) might not be the exclusive limiting factors (Pond et al. 1996; Lee et al. 1999; Broglio et al. 2003; Maps et al. 2005; Vargas et al. 2006; T. Wichard et al. 2006, submitted, Ph.D. Thesis). The algal species such as TR or PM that provide high amounts of polyunsaturated fatty acids results in high EPR and HS values (TR, PM: Table 1, see also example *Thalassiosira weissflogii*, *Isochrysis* sp.: Arendt et al. 2005). However, a recent study by Wichard et al. (2007) found a rapid depletion of polyunsaturated fatty acids in PUA-producing diatoms (TR) upon cell damage. Supplementation of algal diets with EPA increases slightly the hatching success of *Temora longicornis*, which suggests a PUFA-limitation caused by fatty acid transformation into, e.g. PUAs.

If diatoms in general were true nutritionally deficient diets, as it might be supposed, egg production should decrease with all diatoms. Our observations show that it did not with TR, NAV, NIT and SK (Table 1; Poulet et al. 2006). In contrast, PL which is known as an adequate food in terms of essential fatty acids (Delaunay et al. 1993; Lacoste et al. 2001) and does not release any PUA (data not shown), exerted severe degradation in oocytes and EPR decrease (Figs. 1, 9, 10). In fact, EPR, HS and AL measured in four different copepod could hardly be correlated to established indicators of nutritional quality, or to suspended organic particulate carbon and nitrogen (Poulet et al. 2006; Vargas et al. 2006). Identification of the chemical compounds, nutritional factors and cell targets involved with the failure of each reproductive factor is thus open to future research.

Certain diatoms can specifically block egg production and hatching success (or both), whereas others do not (Ban et al. 1997). Besides *T. rotula*, favourable diets could be hardly found among the single species tested during the 2003–2004 survey (Groups I–IV: Table 1). Therefore, copepod populations cannot be durably sustained if one reproductive factor is impaired by a diatom bloom, which lasts more than several weeks. It seems that diatoms mixed with dinoflagellates and/or microzooplankton preys, corresponding to high food diversity, are more favourable to copepod reproduction than single or mixed diatom diets (Lee et al. 1999; Kang and Poulet 2000; Turner et al. 2001; Vargas et al. 2006). Inhibitory mechanisms (1, 2: Fig. 11) are shared with non-diatom species (Table 1, Fig. 1; Lacoste et al. 2001; Ianora et al. 2004b), suggesting that chemical impacts on copepod reproduction might be rather common in the field. Thus, identification of chemical compounds involved in mechanisms (1) and (2) is challenging because inhibition of copepod reproduction by deleterious phytoplankton species is important in ecological terms.

These processes are not observed when food diets are satisfactory. Several studies have reported satisfactory properties of field diets (Irigoien et al. 2002; Castellani and Altunbas 2006; Koski 2007) and it will be a challenge to systematically address positive and adverse effects in bioassay guided approaches. Reproductive inhibition can be sporadic (Halsband-Lenk et al. 2005) or seasonal (Ianora et al. 2004a; Vargas et al. 2006) during the phytoplankton succession. With *C. helgolandicus*, the inhibition of oocyte maturation and egg production interferes significantly the reproductive success at the weekly, monthly and annual scales in the field (Poulet et al. 2006; T. Wichard et al. 2006, submitted, Ph.D. Thesis). The demographic deficit may be even increased when hatching success and/or larval development in addition to egg production are impaired (Miralto et al. 2003; Ask et al. 2006; Vargas et al. 2006). However, we still do not understand the demographic influence exerted by the combined inhibitory mechanisms (1) and (2) on population recruitment and how copepods adapt to survive in nature.

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