# The effects of eight single microalgal diets on sex-ratio and gonad development throughout European flat oyster (*Ostrea edulis* L.) conditioning

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**Abstract:**

To determine the effects of food quality on *Ostrea edulis* reproduction, European flat oysters were conditioned during two sets of experiments, carried out in spring and autumn, during 40 days at 19 °C, in 50 l transparent flow-through tanks, in triplicate, and fed constantly at 900 $\mu m^3 \mu l^{-1}$, with eight different types of microalgae. Four species were fed per group of trials: *Isochrysis affinis galbana*, *Chaetoceros gracilis*, *Skeletonema marinoï*, and *Tetraselmis suecica* were fed to flat oysters in the first set; whereas *Rhodomonas salina*, *Thalassiosira weissflogii*, *Thalassiosira pseudonana*, and *Pavlova lutheri* were provided during the second set of experiments. At the beginning and end of both conditioning periods, oysters were sampled and processed for histology analysis for each diet. Each oyster was classified for its sex and gonad development stage. Oysters fed *S. marinoï* and *C. gracilis* exhibited the highest ratio of hermaphrodites with 96 and 77% respectively, whereas those fed *T. suecica* showed the lowest hermaphrodite percentage, 59%. When oysters were conditioned throughout the second set of experiments with four other species, oysters fed *R. salina* and *T. weissflogii* exhibited 83 and 87% of hermaphrodites. Regardless of the diet, a gonad development occurred during the first set of experiments with ≥ 60% ripe oysters (stage 3) and spawned oysters (stage 4). In the second set of trials, oysters fed *R. salina* and *T. weissflogii* were highly mature with 90% and 75% of stage 3 and stage 4 respectively; whereas those fed *P. lutheri* showed low maturation with only 17% of ripe oysters.

**Highlights**

► We examine effects of eight single diets in gonadic development of *O. edulis* ► At the end of conditioning, large number of hermaphrodites were observed ► *C. gracilis*, *S. marinoï*, *R. salina* and *T. weissflogii* are good diets ► *T. suecica* and *P. lutheri* are poor diets for *O. edulis* broodstock conditionig

**Keywords:** *Ostrea edulis* ; Broodstock conditioning ; Gametogenesis ; Microalgal diets

**Introduction**

From the 70s to the 80s two successive diseases affected *Ostrea edulis* production in France and the population dropped from 20,000 t to 1,000–1,500 t $y^{-1}$ nowadays (Buestel et al., 2009). After diseases extension this situation was quite similar for most countries in Europe (Laing et al., 2005) where the flat oyster population has never recovered. In this context, except in some limited free diseases areas (e.g. Scotland, North, Ireland, Norway, and Denmark) flat oyster farming consists in improving oyster growth before the fateful limit of 3 years old or equivalent size and, accordingly, *O. edulis* production in Europe is constrained.

However, progress has been made in breeding for diseases resistance. Currently, a selective breeding program is, accordingly a possibility to enhance flat oyster farming as earlier shown for *C. virginica* (Ford et al., 1990). Such targeted genetic orientation, however, will not be feasible until the difficulty inherent to a lack of fully reliable methods in hatchery for this species is overcome. Indeed in the hatchery, unexplained mortalities have often been reported during larval rearing on day 8 and post-settlement (Laing et al., 2005). Hatchery methods are now relatively well known for many molluscs (e.g. *Crassostrea gigas*: Utting and Spencer, 1991, *Ruditapes philippinarum*: Helm and Pellizzato, 1990). Despite indisputable know-how, mainly due to pioneer works (Walne, 1974), the state of the art in hatchery rearing of *O. edulis* remains clearly insufficient to support reliable seed production, probably because of a lack of updated, detailed knowledge of the biology of this species.

We therefore focussed on the effects of food on *O. edulis* broodstock conditioning because in this larviparous species maternal effects have been shown to affect larval

growth and survival (Berntsson et al., 1997). In most of molluscs reproduction, temperature has been considered as the key factor controlling reproduction in the natural surrounding (da Silva et al., 2009.) as well as in controlled condition (Wilson et al., 1996). Whereas the effect of food has been recognized more recently (Millican and Helm, 1994; Utting and Millican, 1997) the influence of diet quality, expressed as the species of microalgae delivered, on *Ostrea edulis* conditioning was however poorly known. We developed accordingly research in that field and showed that mixed diets increased fertility and improved subsequent larval development (González-Araya et al., 2012a). Moreover by means of physiological and biochemical studies we showed that *Chaetoceros gracilis*, *Skeletonema marinoï* and *Rhodomonas salina* were highly ingested, assimilated, absorbed and well allocated in all flat oyster tissue including gonads (González-Araya et al., 2011, 2012b). But the effects of different microalgae diet on fine reproduction process (microscopic evolution of gonadic tissues) has not been analysed yet and the aim of the present work is accordingly to study the influence of food on sex dominance and gametogenesis evolution during conditioning in *O. edulis.*

**Material and methods**

*Broodstock conditioning*

We previously showed that a mixed diet was more efficient that single diets on flat oyster fecundity (expressed as number of larvae released: González-Araya et al., 2012a). To define the best microalgae combination we decided to perform trials with single diets to evaluate, firstly on a physiological point of view, which was the most ingested and

digested microalgae, and secondly, through fatty acid and sterol transfer (from microalgae to oyster), which was the most assimilated diet in the gonads. To valid this approach a study of reproduction was carried out in parallel. Lastly, based on the knowledge of some specific biochemical component requirements, the two most physiologically and biochemically efficient microalgae should be associated hypothesising that they were the best diets for conditioning. This strategy, based on the evaluation of different single diets, allowed us to design 8 feeding trials; whereas based on bi-specific assemblage, 32 feeding trials, corresponding to an association of a diatom and a flagellate, were necessary, and thus, extremely difficult to manage. For such similar reason and to respect statistic procedures (triplicate condition per diet), it was necessary to perform such experiment at two different periods of the year.

A total of 720 flat oysters, originated from Brittany, were conditioned during 40 days at 19 °C, in flow-through tanks and fed constantly at 900 $\mu m^3$ $\mu l^{-1}$. Eight different microalgae diets were delivered as single food to oysters, in two sets of experiments due to technical limitation (four microalgae tested within each set), to study feeding physiological needs and its incidence on reproduction process. **All different algae were supplied at the same bio-volume.** 18-month-old *O. edulis* (≈ 5 cm length and 0.5 g meat dry weight), originated from Bay of Cancale (North Brittany, France) were distributed homogeneously, in February 2008, in translucent 50 L tanks (30 oysters per tank for an equivalent biomass), in triplicate for each of the four single diets used here. They were previously treated in chloramphenicol at 8 mg l$^{-1}$ to limit any development of *Vibrios* and the initial spring condition corresponds to the oyster sampled prior to the distribution. The first experimental period was assessed using four microalgae: *Isochrysis affinis galbana*

(T: CCAP 927/14), *Chaetoceros gracilis* ($C_g$: UTEX LB2658), *Skeletonema marinoï* ($S_m$: CCAP 66/4), *Tetraselmis suecica* ($T_s$: CCAP 1077/3).

In August 2008, *O. edulis* aged 18 months (≈5 cm length and 0.5 g flesh dry weight), originating from Bay of Quiberon (South Brittany, France) were submersed, at 5 m depth, for 1 month, in mesh bags tied to trestles in the Bay of Brest. They were then returned to the quarantine area of the Argenton hatchery, where they were maintained at 14 °C for an additional month, during which they were treated for a week with chloramphenicol. Thereafter, seawater temperature was increased by 1 °C weekly and, at beginning of October 2008, the flat oysters were transferred to translucent 50-l tanks where they were distributed homogeneously (30 oysters per tank, corresponding to an equivalent biomass of ≈1 kg total weight and 16 g dry flesh weight). During this pre-conditioning period oysters were fed a mixed diet of T. Iso and *C. gracilis* used routinely in Argenton to feed *C. gigas* at different stages of development (Ben Kheder et al., 2010). The initial autumn condition corresponds to the oyster sampled prior to their distribution in individual tank. Triplicate tanks were set up for each of the four single diet species tested here during this second experimental period: *Rhodomonas salina* ($R_s$: CCAP 978/24), *Thalassiosira weissflogii* ($T_w$: CCAP 1085/1), *Thalassiosira pseudonana* ($T_p$: CCAP 1085/3) and *Pavlova lutheri* ($P_l$: CCAP 931/1

At the end of both conditioning periods, oysters were sampled from each tank and processed for histological analysis.


*Histological analysis*

Each oyster was carefully opened, and a 5-mm thick section of tissue was excised parallel to the anterior-posterior axis between the labial palps and the posterior adductor muscle. They were thereafter fixed in Davidson's solution and embedded in paraffin. Tissue sections achieved in this manner contain gametes representative of the whole gonad. Finally, 5-µm thick sections were stained with Harris's haematoxylin and eosin (Howard and Smith, 1983). Reproduction was studied on histological sections and each oyster was classified according to sex category and gonad development based on a scale detailed by da Silva et al. (2009). The sex was assigned as following: Indeterminate (I), when material had just collapsed or empty follicles are visible (Fig. 1a); male solely (M), when follicles contained only male gonadic material (Fig. 1b); female solely (F), when follicles contained only female gonadic material (Fig. 1c); hermaphrodite (H), when female or male gonad material were observed, separately of prevalence sex (Fig. 1d-1e-1f).

Then a score of 0 to 4 was assigned to these figures with 0 = inactive gonad and 4 = empty due to spawning). Five stages per functional sex of gonad development were considered as following:

- Inactive or resting gonad (0) (Fig. 1a): no evidence of ripe gametes development. The gonad is dilated and empty; follicles are located between mantle and digestive gland surrounded by abundant connective tissue.

- Early gametogenesis (1) (Fig. 1b): gonad follicles are more spread into the connective tissue with ovogonia and spermatogonia mostly attached to the follicle wall. In the male part, there are primary and secondary spermatocytes. In the female part, developing oocytes are attached to developing lines.

- Advanced gametogenesis (2) (Figs. 1c, 1d): gonad follicles are larger than in the previous phase, but connective tissue is still present. In the male section the

development of few spermatogonia still occurred, but sparmatocytes and spermatid balls are dominant; in the female section, oocytes in vitellogenesis are dominant, while oocytes in post-vitellogenesis are sparse.

– Ripe gonad (3) (Figs. 1e, 1f): juxtaposed large follicles occupied the entire area between the mantle and digestive gland. Both male and functional female developing lines, follicles contained gametes, abundant spermatozoa balls and mature oocytes.

– Spawned (4): gonad follicles are smaller than in the previous phase. Gametes have been released and residual mature ooocytes and sperm balls could be observed in the follicle lumen. Phagocytes are often observed in the follicle lumen.

When hermaphrodites were detected, the subcategory of one sex predominance was not considered, because the aim of this experience was to study the overall effects of mono-specific diets on gonadic development without detailing hermaphrodite aspects.


*Statistical analysis*


Comparison of oyster distribution in gonad condition classes (sex and gonad development) between conditioning diet and sex category were analyzed using a Chi-square test of independence. The same procedure was applied to test the association of gonad condition classes and season of conditioning, in which the sex or gonad development was organized into columns, and the *O. edulis* diets into rows. Statistical analysis was performed using the software STATISTICA (version 8.0). Differences were considered statistically significant when $p$ values were lower than 0.05.

**Results**

Oysters fed *S. marinoï* and *C. gracilis* during spring conditioning exhibited the highest percentage of hermaphrodites with 96 and 77% respectively; whereas those fed *T. suecica* showed the lowest hermaphrodites ratio (59%: Table 1). In autumn when oysters were fed *R. salina* and *T. weissflogii* 83 to 87% hermaphrodites were recorded whereas those fed *T. pseudonana* and *P. lutheri* led to 58 and 33% hermaphrodites respectively. However, the highest proportion of female was founded in oysters fed *P. lutheri* (33%) and the lowest in those fed *S. marinoï* (0%: Table 1). Except in oysters fed *R. salina*, male was observed in all samples (Table 1). High percentages of indeterminate were observed in the two initial conditions, spring (21%) and especially autumn (67%) as well as in oysters fed *T. suecica* (24%) and *P. lutheri* (23%).

Regardless diet and season, gonadic development occurred. At the end of spring conditioning the marked differences were observed ($\chi^2$= 6.02; df=4; P< 0.001) with more than 60% of oysters were ripe (stage 3) or have recently spawned (stage 4) (Fig. 2). The highest value of recently spawned individuals was recorded in oysters fed T-Iso; whereas those fed *S. marinoi* and *C. gracilis* showed the highest values of ripe gonads (70 and 55% respectively). Those receiving *T. suecica* and T-Iso exhibited 38% and 31% of ripe gonads respectively. Apart from initial condition, early gametogenesis was only observed in oysters fed *T. suecica* (3%: $\chi^2$ = 26.17; df = 1; P = 0.048) (Fig. 2).

In autumn, the highest values of stages 3 and 4 were observed in oysters fed *R. salina* (> 90%) and *T. weissflogii* (> 75%) whereas the lowest value was observed for those fed *P. lutheri* (17%: $\chi^2$ = 82.14; df = 2; P< 0.001). Initial and advanced gametogenesis (stages 1

and 2) were observed in oysters regardless diets, whereas stage 0 (indeterminate) was undetected in oysters fed *R. salina*. At the initiation of this fall conditioning period, 33% of recently spawned oysters and 67% of indeterminate were found ($\chi^2$= 8.85; df = 4; P = 0.048).

**Discussion**

At the end of conditioning periods, most of *O. edulis* examined in spring and autumn were hermaphrodites, excepted for initial condition in autumn with 67% of indeterminate. The presence of large number of hermaphrodites, as well as small percentage of males and females may indicate that, gametogenic phase followed the next one without complete resorption as already reported (Siddiqui and Ahmed, 2002).

Whereas temperature effects on gonad development and spawning are nowadays well known (e.g. Mann, 1979; Helm et al., 2004), the influence of food on the reproductive pattern of bivalves which includes growth, maturation, spawning, resorption and resting period are less documented (Kang et al., 2000). The occurrence of greater proportion of ripe gonad (stage 3) and spawning (stage 4) in oysters fed *C. gracilis*, *S. marinoi*, *R. salina* and *T. weissfloggi* indicate that gametogenesis was more rapid than with other diets used. This result could be explained by specific biochemical allocations in gonads. Despite, the absence of correlation between fatty acids concentration and gonadic development (González-Araya et al., 2011) it has been showed that cholesterol concentration in *C. gracilis* and *S. marinoi* were higher than in T-Iso and *T. suecica*. For autumn conditioning, similar concentrations of brassicasterol and cholesterol in gonads of oysters fed *R. salina* and *T. weissflogii* have been already reported (González-Araya et al., 2012a). The role of

cholesterol in mollusks gametogenesis is still unknown but bivalves have a limited capacity for cholesterol synthesis or bioconversion of phytosterols into cholesterol (Napolitano et al., 1993; Kanazawa, 2001). Phytosterols present in oysters reflect accordingly ingested microalgae. Different microalgae synthesize specific phytosterols (Palacios et al., 2007): for example, brassicasterol has been found in high concentrations (90%) in *Isochrysis sp.* (Volkman et al., 1981), whereas 24-methylenecholesterol and also cholesterol could be recorded in diatomophyceae such as *Chaetoceros sp.*, *Skeletonema sp.*, *Thalassiosira* sp. (Soudant et al., 1998; González-Araya et al., 2012b).

On eight microalgae commonly used in hatchery four of them (*C. gracilis*, *S. marinoï*, *R. salina* and *T. weissflogii*) promoted a better and faster *O. edulis* gonadic development. In contrast, oysters fed *T. suecica* and *P. lutheri* showed the lowest gametogenesis and are accordingly not recommended for broodstock conditioning. T-Iso occupied an intermediate position and combined with a diatom could represent an efficient diet for *O. edulis* broodstock conditioning but never as single diet.

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**List of tables:**

**List of figures:**

Table 1.

| Sex category | Spring conditioning | | | | | Autumn conditioning | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | I | T | $C_g$ | $S_m$ | $T_s$ | I | $R_s$ | $T_w$ | $T_p$ | $P_l$ |
| Indeterminate | 21.4 | 0.0 | 4.5 | 0.0 | 24.1 | 66.7 | 0.0 | 6.5 | 12.1 | 23.3 |
| Male | 14.3 | 15.4 | 13.6 | 4.3 | 10.3 | 16.7 | 0.0 | 3.2 | 6.1 | 10.0 |
| Female | 0.0 | 15.4 | 4.5 | 0.0 | 6.9 | 16.7 | 17.2 | 3.2 | 24.2 | 33.3 |
| Hermaphrodite | 64.3 | 69.2 | 77.3 | 95.7 | 58.6 | 0.0 | 82.8 | 87.1 | 57.6 | 33.3 |

**I**: Initial condition; oyster broodstock fed: T: T-Iso; $C_g$: *C. gracilis*; $S_m$: *S. marinoi*; $T_s$: *T. suecica*; $R_s$: *R. salina*
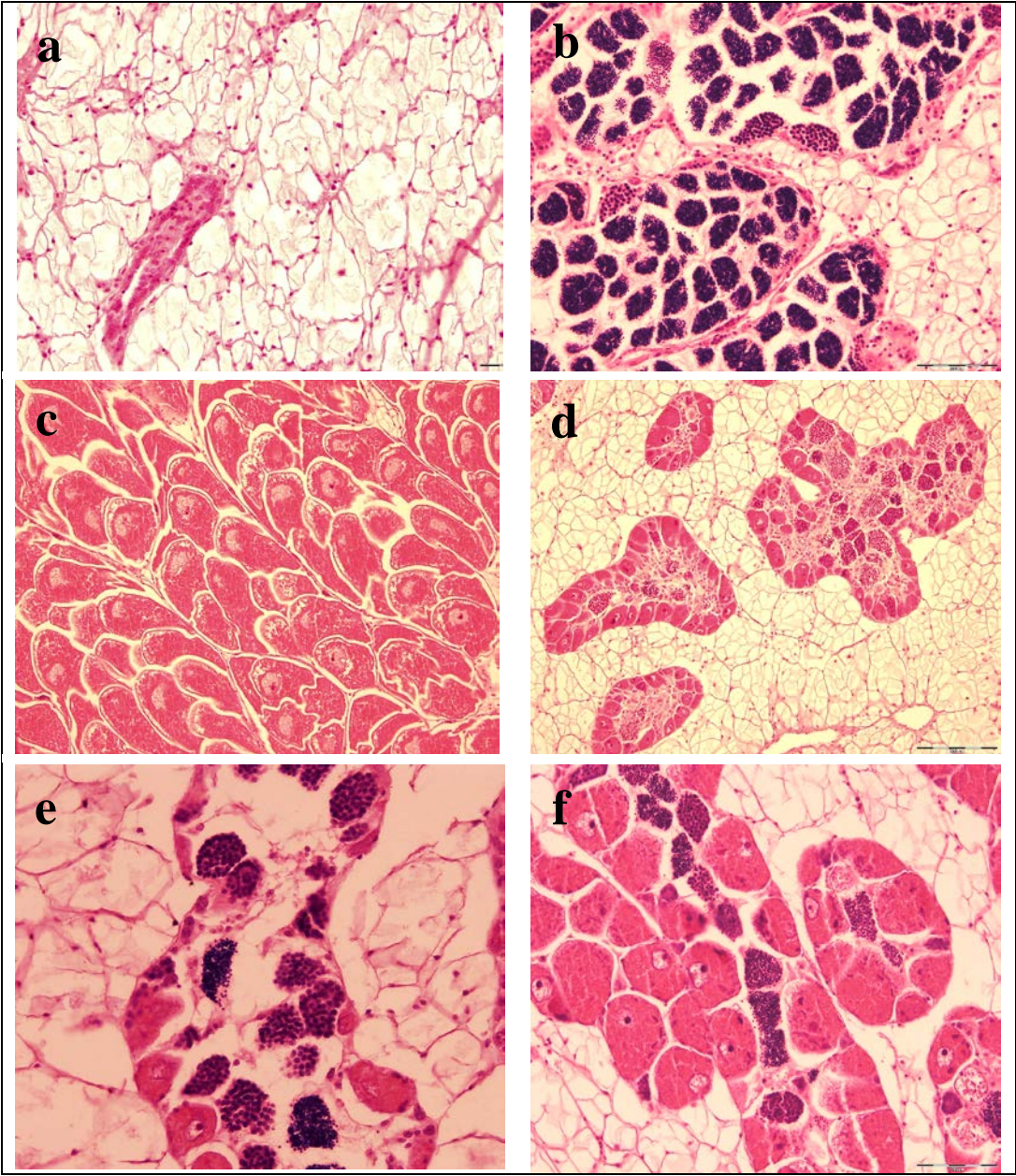$T_w$: *T. weissflogii*; $T_p$: *T. pseudonana*; $P_l$: *P. lutheri*.

Figure 1

Figure 2