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RESEARCH ARTICLE

Acquisition of particle processing capability in juvenile oyster *Crassostrea gigas*: ontogeny of gill mucocytes

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Abstract Acquisition of particle processing capability in postlarval oysters depends upon the structural development of the pallial organs, as well as the development of cilia and mucocytes used (either directly or indirectly) in particle capture and transport. Mucocyte mapping was therefore used to identify mucocyte types and distributions throughout gill development in juvenile oyster *Crassostrea gigas* (Thunberg 1793) specimens from 2.9 mm to 2.4 cm in shell length. Three categories of gill filaments were identified: apical, lateral and principal filaments, corresponding to filament location or future location in gill plicae. Mucocyte densities were recorded per linear μm (l μm) of frontal surface, and converted to potential total volumes, using the mean volumes of each of the two major mucocyte types: acid mucopolysaccharide (AMPS)-mucocytes and mixed mucopolysaccharide (MMPS)-mucocytes. While AMPS secretions were dominant up to 1.0 cm (flat homorhabdic gill, to semi-heterorhabdic differentiation and plication), MMPS secretions increased progressively, dominating in 2.4 cm and adult specimens (fully heterorhabdic and plicated). Mucus composition, and hence mucus viscosity, thus appears to evolve in relation to the degree of enclosure of the gill frontal surfaces. Total (AMPS + MMPS) potential mucus secretion increased allometrically with juvenile growth, characterized by a sharp increase between 10 and 24 mm shell length, suggesting a marked improvement in particle processing capability. Mucocyte distributions on the gill were heterogeneous from the onset of heterorhabdic differentiation (7.5 mm): the apical filaments of the plicae contained much greater mucocyte total volumes, compared to the lateral and principal filaments. In addition to mucus composition, total potential mucus volume thus also evolved in relation to the degree of enclosure of the gill frontal surfaces. These results show that functional specialization in mucocyte distribution precedes the complete anatomical heterorhabdic differentiation. The completely functional adult gill system is thus attained in 2.4 cm juveniles. This information should be of use in understanding the dynamics of juvenile feeding, growth, and mortality, both in natural systems and in rearing operations.

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Introduction

In coastal marine ecosystems, the decline in natural oyster populations has been accompanied by an opposite increasing trend in domesticated farming, currently accounting for approximately one-third of the total world bivalve production. Following widespread introductions, *Crassostrea gigas* has become the most ubiquitous and abundant oyster species worldwide, both in and out of culture operations (FAO 2005; Ruesink et al. 2005). These introductions have provoked fundamental changes in ecosystem function (Ruesink et al. 2005). The impact of this species on coastal ecosystems and economies has spurred research

on its biology, and in particular its interactions with the seston component (Riera and Richard 1996; Barillé et al. 1997; Beninger et al. 2006).

The mechanisms of particle treatment in the genus *Crassostrea* have been the focus of several contemporary studies, mostly concerned with the adult life stage (Ward et al. 1993, 1994, 1998; Beninger and Dufour 1996; Barillé et al. 1997, 2000; Beninger and St-Jean 1997a; Beninger and Veniot 1999; Cognie 2001; Cognie et al. 2003; Beninger et al. 2005), in which the structure of the processing organs is well-known—in particular the heterorhabdic pseudolamellibranch gill, unique to the Ostreidae (Atkins 1937a, b, 1938; Nelson 1960; Galtsoff 1964; Ribelin and Collier 1977; Barillé 1994; Eble and Scro 1996; Dufour and Beninger 2001). The role of mucocytes in particle processing on the gill has been detailed and underscored for the various bivalve species studied, representing the principal gill types (Beninger et al. 1992, 1993, 1997), including the oyster (Ward et al. 1993; Beninger and Dufour 1996; Beninger and St-Jean 1997a), through correlation with direct endoscopic observations. Although important functional changes in particle processing have been shown to occur in early life stages (Wilson 1980; Baker and Mann 1994a, b), the understanding of the underlying feeding mechanisms has been hampered by a lack of knowledge of the structure of the particle-processing organs. The recent structural studies of gill and mantle development in brooding (*Ostrea edulis*—Chaparro et al. 2001) and non-brooding species (*C. gigas*—Cannuel and Beninger 2006; Beninger and Cannuel 2006) have both identified critical stages in pallial organ development, and opened the way to further research on the mechanisms of particle processing in early life stages. Although the small size of specimens in these stages makes fine-scale direct functional observations difficult, it is possible to deduce fundamental principles of particle processing mechanisms using indirect techniques such as mucocyte mapping (Beninger et al. 1993; Beninger and Dufour 1996; Beninger and St-Jean 1997a, b; Dufour and Beninger 2001; Beninger et al. 2003; Dubois et al. 2005). Mucocyte mapping has been shown to be particularly informative with respect to the fine-scale functioning of the adult oyster gill (Beninger et al. 2005).

The present study reports on the ontogenetic changes in mucocyte total volumes and distributions on the *C. gigas* gill during development of the juvenile life stage, and relates these changes to functional capacities of particle processing.

Materials and methods

For the purposes of clarity and consistency, the term ''juvenile'' is used here to designate individuals ≥2.9 mm (Beninger and Cannuel 2006). This size was chosen as the developmental starting point, since the gill is homorhabdic up to this point; differentiation to the heterorhabdic condition and plication begin above this size.

Juvenile collection and histological processing

Juvenile *C. gigas* were obtained from the Vendée Naissain commercial hatchery (Baie de Bourgneuf 47°5′S, 2°5′W; France) in September and December 2003. Four stages were studied, corresponding to four commercial sifter-sizes and to successive developmental stages of the gill (Fig. 1 and Cannuel and Beninger 2006). Fifteen specimens per size class were first narcotized in ascending concentrations of $MgCl_2$ up to 7.5% (Veniot et al. 2003; Cannuel and Beninger 2006), and then fixed in aqueous Bouin's solution. After a minimum of 48 h fixation, they were rinsed under running tap water for 24 h, dehydrated, and embedded in paraffin. Transverse 5 μm sections of the whole specimens were made in the gill area for each specimen, and stained using the alcian blue-Periodic acid-Schiff (PAS) protocol (Beninger et al. 2005), modified as follows: 1% alcian blue pH 2.5 (1 min), 1% periodic acid (1 min), Merck Schiff reagent (3 min), sodium metabisulfite (3 × 3 min). The resulting mucocyte secretion colours were determined according to the Pantone® colour codes previously used in adult *C. gigas* gills (Beninger et al. 2005).

It was necessary to verify frontal surface width homogeneity within each individual, size class and filament type; frontal surface width was also used as a measure of filament growth through gill development. Twenty to 30 filament widths per filament type (ordinary or principal filament) were measured, in histological sections, for 5 individuals per size class. Due to the extremely small widths of the living filaments, it was not possible to determine a processing shrinkage coefficient; however, shrinkage has been shown to be constant for the adult gill (Beninger et al. 2005), such that the values reported here for the different juvenile sizes may be compared without bias from variable shrinkage.

Mucocyte counts

Mucocyte counts were performed on 30 haphazardly-chosen filaments in 5 oyster juveniles, in order to establish their relative abundances on the component

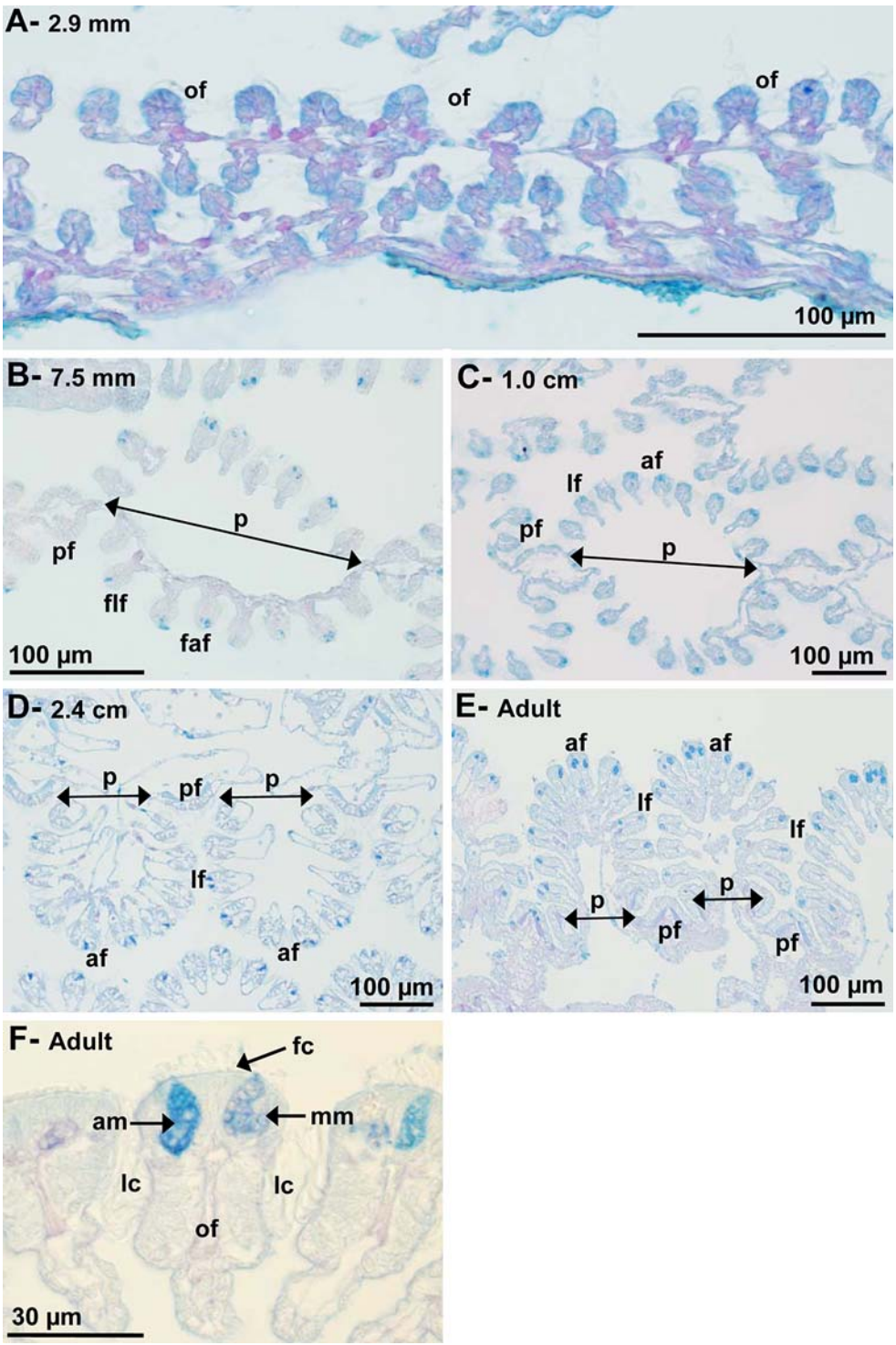

Fig. 1 *Crassostrea gigas*. Photomicrographs of histological sections of juvenile and adult oyster gills, showing mucocyte types and locations in relation to major steps in gill development. **a** 0.29 mm juvenile: flat homorhabdic gill; **b** 7.5 mm juvenile: slightly plicate gill, onset of principal filament (and thus heterorhabdic) differentiation; **c** 1.0 cm juvenile: moderately plicate gill, continuation of heterorhabdic differentiation; **d** 2.4 cm juvenile: deeply plicate heterorhabdic gill; **e** adult: deeply plicate heterorhabdic gill; **f** Adult: detail of a gill ordinary filament section showing the two mucocyte types: acid mucopolysaccharide (AMPS)-mucocytes and mixed mucopolysaccharide (MMPS)-mucocytes. PAS-alcian blue protocol. *af* apical filament, *am* AMPS-mucocyte, *faf* future apical filament, *fc* frontal cilia, *flf* future lateral filament, *lc* lateral cilia, *lf* lateral filament, *mm* MMPS-mucocyte, *of* ordinary filament, *p* plica, *pf* principal filament. For *af*, *faf*, *flf*, *lf*, *pf* component gill unit used for mucocyte counts

filaments of the gill. At sizes >2.9 mm, three counting zones were distinguished corresponding to three filament categories: (1) ordinary apical and (2) ordinary lateral filaments of the plicae, and (3) principal filament troughs (Fig. 1). Principal filaments are formed by the ontogenetic fusion of three ordinary filaments, corresponding to the two lateral walls (the ''transitional filaments'' of Ridewood 1903; Galtsoff 1964; Eble and Scro 1996) and the trough (Beninger and Dufour 1996; Cannuel and Beninger 2006). To facilitate reading, principal filament troughs will hereafter be termed ''principal filaments''.

Mucocyte counts were performed at 200×, using a digital camera and computer screen. Images were

stored using LUCIA G® software (Nikon), pending counts. Mucocyte numbers were recorded per linear μm (l μm) of frontal surface. The extremely small widths, and hence small frontal tract widths, of juvenile gill filaments precluded fine-scale mucocyte mapping within the frontal tracts (Beninger et al. 2005).

In order to evaluate the instantaneous total volumes of mucus contained in mucocytes, 30 mucocytes of each mucocyte type were measured and converted to volumes for each juvenile size class. All mucocytes were ellipsoids in form, and the corresponding volumes were calculated. Mean volumes were then calculated for each mucocyte type and each juvenile size class; these values were multiplied by mean mucocyte numbers to obtain the mean total mucocyte volumes. The relative proportions of AMPS in the mucus resulting from the mixing of the different mucocyte type secretions on the filament surface, were calculated for each size class (2.9, 7.5 mm, 1.0, 2.4 cm) and each filament type (apical, lateral or principal filament) according to Beninger et al. (2005). Calculations were performed for undifferentiated filaments in 2.9 mm specimens.

Hypotheses and statistical analyses

The following two sets of hypotheses were formulated:

1.

H_0: No significant difference in mucocyte types and total volumes between the juvenile size classes. Corollary: there are no ontogenetic changes in mucocyte types and total volumes which would affect particle processing function.

H_1: Significant difference in mucocyte types and total volumes between the juvenile size classes. Corollary: ontogenetic changes in mucocyte types and total volumes occur during juvenile development, which may affect particle processing function.

2.

H_0: No significant difference in mucocyte types and total volumes between apical, lateral and principal filaments. Corollary: no ontogenetic changes occur in the roles of the ordinary and principal filaments which would affect particle processing in developing juveniles.

H_1: Significant difference in mucocyte types and total volumes between apical, lateral and principal filaments. Corollary: ontogenetic changes occur in the roles of the ordinary and principal filaments, which may affect particle processing in developing juveniles.

Parametric or non-parametric one-way ANOVA (tested factor vs. shell size filament type, and mucocyte type) were performed on the mean mucocyte volumes, total volumes, and filament frontal surface width data, depending on normality and heteroscedasticity of data, with $\alpha = 0.05$.

Results

Mucocyte types and volumes

Two types of mucocytes were most frequently observed: acid mucopolysaccharide (AMPS)-containing mucocytes, and mixed (acid + neutral) mucopolysac-

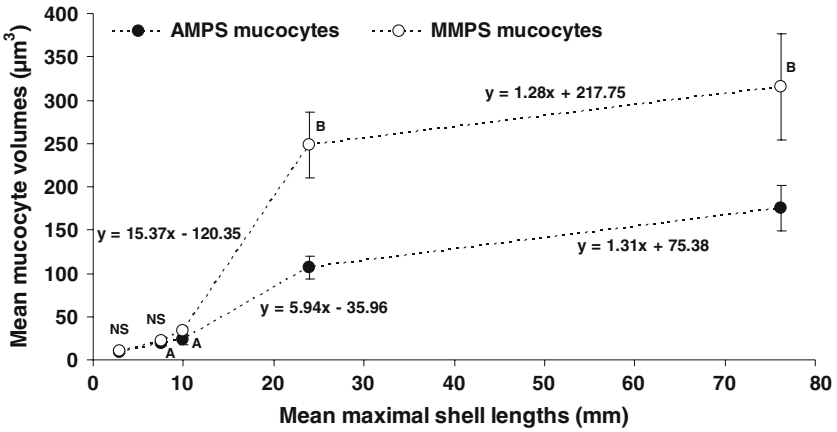

Fig. 2 *Crassostrea gigas*. Mean mucocyte volumes (μm³) of acid mucopolysaccharide (AMPS)-mucocytes and mixed mucopolysaccharide (AMPS)-mucocytes for each shell size examined. Adult data from Beninger et al. (2005). Means ± 95% CI (confidence interval). *NS* values of AMPS and MMPS not significantly different ($P = 0.648$ and 0.472 for 2.9 and 7.5 mm juveniles, respectively). Values of AMPS or MMPS with the same *upper case letter* do not differ significantly ($P = 0.515$ for *A*, $P = 0.147$ for *B*). Linear equations are given for the (10–24 mm) and (24–76 mm) intervals

charide (MMPS)-containing mucocytes (Fig. 1). Neutral mucopolysaccharide (NMPS)-containing mucocytes were rarely observed and hence not counted.

Mean mucocyte volumes increased unequally for the two mucocyte types throughout gill development: from similar volumes in the smaller specimens, MMPS-mucocytes progressively became larger than AMPS-mucocytes, with significant differences from a length of 1.0 cm. A marked increase in both AMPS- and MMPS-mucocyte volumes occurred between 10 and 24 mm, after which they leveled off to the adult condition (Fig. 2).

Frontal surface width homogeneity

Frontal surface widths were homogeneous for each filament category and for each size class examined (Fig. 3). Mean filament width values were thus used for calculation of all mean mucocyte densities, and subsequently, total volumes. Linear equations were calculated for the major phases revealed by the graphs; as they comprise few shell size categories, linear regressions were not possible.

An increase in both ordinary, and to a greater extent principal, filament frontal surface width was recorded throughout gill development (Fig. 3; $P \leq 0.001$). A sharp increase in principal filament frontal surface width was observed between 10 and 24 mm, with a more gradual rate of increase to 55.42 µm wide in adults (Fig. 3). In contrast, the ordinary filament frontal surface enlarged more gradually during juvenile growth ($y = 2.03x - 1.08$ vs. $y = 0.55x + 9.4$). Between 24 and 76 mm, the rate of increase of ordinary filament frontal surface widths was slight, as was observed for the principal filaments.

Mucocyte total volumes

In all filaments, AMPS mean total volumes dominated, with respect to MMPS in juveniles from 2.9 mm to 1.0 cm (Fig. 4a–c). A marked increase in AMPS was observed in the apical and lateral filaments from 2.9 to 7.5 mm ($P \geq 0.001$ and $P = 0.004$), whereas the MMPS remained stable ($0.12 < P < 0.74$). In 7.5 mm and 1.0 cm juveniles, no significant difference was observed between AMPS and MMPS mean total volumes in differentiating principal filaments ($P = 0.163$ and $P = 0.056$; Fig. 4b, c). In 2.4 cm juveniles and adults, MMPS mean total volumes dominated with respect to AMPS in apical, lateral and principal filaments (Fig. 4d, e).

From 7.5 mm, cumulative (AMPS + MMPS) mean total mucocyte volumes were greater in the apical filaments with respect to the lateral and principal filaments. Apical filaments contained more than 1.4–2.2 and 3.1–4.2-fold larger cumulative total mucocyte volumes, compared to the lateral and principal filaments, respectively (Fig. 4b–e). In adults, apical filaments contained 2.2 and 3.7-fold the cumulative total mucocyte volumes of the lateral and principal filaments, respectively (Fig. 4e).

Relative AMPS potential secretion

Acid mucopolysaccharide were dominant in the total mucus volume potentially secreted at the frontal surface of the homorhabdic gill filaments in 2.9 mm juveniles, representing approximately 76% (Table 1). In the larger sizes, the general trend showed a decrease in the proportions of AMPS in the total mucus volume throughout gill development, together with an increase in MMPS (Table 1). However, AMPS percentages in

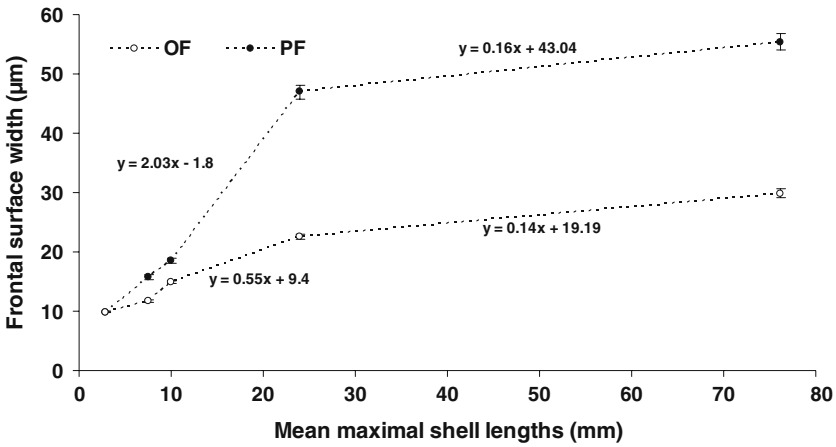

Fig. 3 *Crassostrea gigas*. Mean frontal surface widths of ordinary (*OF*) and principal (*PF*) filaments in juveniles and adults (means ± 95% CI). Widths of *OF* and *PF* significantly different ($P \leq 0.001$) within and between size classes. Linear equations are given for the (10–24 mm) and (24–76 mm) intervals. No principal filaments exist in the 2.9 mm homorhabdic stage

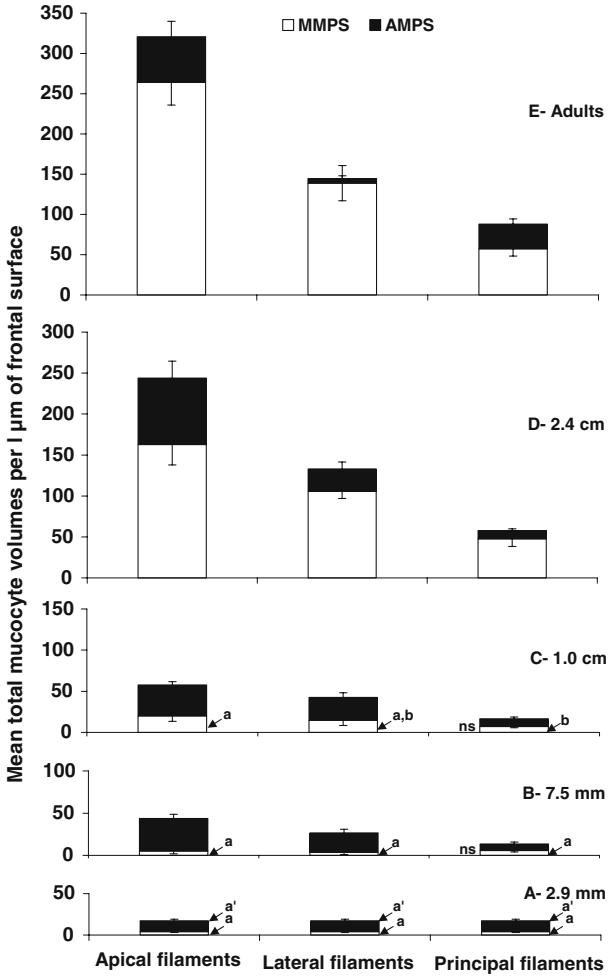

Fig. 4 *Crassostrea gigas*. Mean total acid mucopolysaccharide (AMPS) and mixed mucopolysaccharide (MMPS)-mucocyte volumes (μm^3) per linear µm (l µm) of frontal surface. Means ± 95% CI (*vertical bars*). **a** 2.9 mm juveniles, **b** 7.5 mm juveniles, **c** 1.0 cm juveniles, **d** 2.4 cm juveniles, **e** Adults (data derived from Beninger et al. 2005 for apical and lateral filaments). Values of AMPS or MMPS with the same *lower case letter* do not differ significantly (horizontal comparison). *ns* Values of AMPS and MMPS not significantly different (vertical comparison)

the total mucus volume varied depending on the filament category considered. Thus, AMPS represented 33% of the potential total mucus volume for the apical filaments, compared to 21% for the lateral filaments and 18% for the principal filaments in 2.4 cm juveniles, a difference of approximately 1.6- and 1.8-fold, respectively (Table 1).

In adults, AMPS represented 17% of the total mucus volume in the apical filaments, 4 and 35% in the lateral and principal filaments, respectively. An increase in AMPS proportions was thus observed in principal filaments at the end of gill development (Table 1).

Table 1 *Crassostrea gigas*. Mean percentage of acid mucopolysaccharides (AMPS) in the total potential mucus secretion volume in the apical, lateral and principal filaments (±95% CI $n = 5$ for juveniles, $n = 14$ for adults), for each size class

Mean shell lengths	Apical filament	Lateral filament	Principal filament
2.9[a] mm	76 ± 7	76 ± 7	76 ± 7
7.5 mm	88 ± 8	86 ± 11	57 ± 12
1.0 cm	66 ± 9	66 ± 12	57 ± 8
2.4 cm	33 ± 8	21 ± 6	18 ± 7
Adults (7.6 cm)	17 ± 6	4 ± 2	35 ± 8

[a] For 2.9 mm juveniles, only ordinary filaments were considered due to the homorhabdic condition of the gill at this stage (Cannuel and Beninger 2006, present study). Adult data derived from Beninger et al. (2005) for apical and lateral filaments

Discussion

Ontogeny of mucus production

The observed evolution of mucocyte sizes throughout gill development shows that mucocytes mature progressively, and that mucocyte types and volumes evolve allometrically, especially between 10 and 24 mm shell length. The levelling-off observed from 24 mm shell length, suggests completion of mucocyte maturation.

The differences observed in mucocyte types and total volumes between the juvenile size classes, lead to the rejection of the null hypothesis $H_0(1)$, and to the acceptance of experimental hypothesis $H_1(1)$: the total volumes of the two mucocyte types, and therefore mucus composition, evolves during juvenile development. The proportion of AMPS in the total potential mucus secretion, representing approximately 75% in 2.9 mm juveniles, decreased and reached 4–18% in adults. Given that AMPS are more viscous than MMPS, the viscosity of the mucus resulting from the mixing of mucocyte secretions at the frontal surface of gill filaments decreased throughout development (Beninger et al. 2005, present study), in proportions related to filament location (apical, lateral or principal filaments). The principal filaments presented the lowest mucus total volumes but the greatest AMPS proportions in the latest stage, as previously observed in *Crassostrea virginica* adults (Beninger and Dufour 1996). The localization of the principal filament mucocytes was extremely specific, being aligned on the median crests of the principal filament troughs, as in *C. virginica* adults (Beninger and Dufour 1996).

The considerable increase in total potential mucus secretion, from the homorhabdic stage at 2.9 mm to adult size (approximately 18-, 8- and 5-fold for the final apical, lateral and principal filaments, respectively),

suggests a general increase in particle processing capability, especially on the apical filament frontal surface. Together with the simultaneous increase in gill filament number and length (Cannuel and Beninger 2006), this may be related to a considerable increase in grazing rates, as observed experimentally in *Ostrea edulis* (Wilson 1980).

The distributional difference observed in mucocyte types and total volumes between apical, lateral and principal filaments lead to the rejection of the null hypothesis $H_0(2)$, and to the acceptance of experimental hypothesis $H_1(2)$: not all the gill filaments play an equal role in particle processing on the gill. A marked change in mucocyte distribution between the three filament categories was observed at 7.5 mm, where the future apical filaments presented approximately twice the total mucus volume compared to the differentiating (non-functional—see Cannuel and Beninger 2006) principal filaments, and 1.5-fold those of the future lateral filaments. From this stage, the homorhabdic gill initiates plication and principal filament differentiation (Cannuel and Beninger 2006, present study). In light of the dominant role of the apical filament on the adult oyster plicae (Beninger et al. 2005), it is thus clear that specific mucocyte distributional differentiation precedes the anatomical heterorhabdic differentiation. To summarize, functional specialization found in the adult occurs for the mucocyte distribution on the three filament categories (apical, lateral and principal), well before complete plica establishment.

While well-suited to the plicate condition of the adult, the increase in mucocyte density on the future apical filament, and not on the future lateral filaments, may indicate a processing capability disparity in 7.5 mm juveniles. This observation is in line with the previously-described critical character of the 7.5 mm stage (Cannuel and Beninger 2006).

Particle processing implications

Studies of particle transport in adult oysters consistently report a bi-directional particle transport on the gill frontal surface, with particles transported ventrally on the ordinary filament plicae, and dorsally on the principal filaments (Atkins 1937a, b; Nelson 1960; Galtsoff 1964; Barillé 1994; Ward et al. 1994, 1998; Cognie 2001; Cognie et al. 2003). Bi-directional transport is assumed to be one of the prerequisites of both quantitative and qualitative particle selection on the gill in adult oysters: particles sent ventrally are initially rejected and particles sent dorsally are initially accepted prior to second-stage selection on the labial palps (Ward et al. 1998; Cognie et al. 2003). While the possibility of bi-directional transport on the undifferentiated ordinary filaments of juveniles (up to 7.5 mm shell length—Cannuel and Beninger 2006) cannot be excluded, this seems rather remote, given the small size of the frontal surface (10 μm in 0.42 mm postlarvae—Cannuel and Beninger 2006), and the prevalence of unidirectional transport on homorhabdic gills.

While the heterorhabdic condition, and its attendant bi-directional transport, may be necessary prerequisites for particle selection on the gill, it has become apparent that different mucus types are associated with different processing tasks, including selection. In conformity with the general pattern observed in bivalve gills (Beninger and St-Jean 1997a), the viscosity of the mucus encountered at the frontal surface of the gill filaments seems to evolve during development in relation to the degree of exposure of the surfaces to the current flow and to the direction of particle transport on the gill frontal surface. The most viscous secretions are found on exposed gill surfaces with counter-current particle transport (ordinary filaments in the homorhabdic condition, apical ordinary filament in the heterorhabdic condition—Beninger et al. 2005, present study). As a rule, lower-viscosity secretions are encountered on semi-enclosed surfaces with forward-current particle transport (Beninger and St-Jean 1997a); the apparently contradictory situation in the oyster principal filament, with a row of AMPS-mucocytes on the crest of the median ridge, has been commented upon previously (Beninger and Dufour 1996), and may be related to processing functions not found in the filibranch heterorhabdic gill type (unpublished data). It is significant that viscous mucus masses are never observed in the dorsal gill tract of the oyster (Ward et al. 1994; Cognie et al. 2003), so these AMPS secretions do not appear to contribute to the flow exiting the principal filaments. Given the dense ciliation accompanying the mucocytes at the crest of the median ridge, this surface appears mucociliary in nature. The visual inaccessibility of the principal filament trough *in vivo* does not allow us to interpret this unique situation among the bivalve gill types.

The overwhelming dominance of AMPS-mucocytes on the frontal surfaces of the gills in the smaller specimens observed (2.9 mm to 1.0 cm) means that mucus arriving at the labial palps is much more viscous than in the adults. Since qualitative particle selection on the labial palps requires fluidization of the arriving mucus masses, either by mechanical action (proposed in *C. virginica*—Newell and Jordan 1983) or by addition of neutral mucopolysaccharides (*Mytilus edulis*—Benin-

ger and St-Jean 1997b), it would be interesting to investigate the selection capacity of individuals at the different developmental stages of the oyster, and relate this to the density and distribution of mucocyte types on the labial palps.

Previous anatomical study of *C. gigas* gill development suggested that the definitive gill structure and functioning was only attained from the 2.4 cm size onward (Cannuel and Beninger 2006). The data of the present study corroborate this interpretation, based on the fundamental differences in mucocyte volumes, filament frontal surface widths, and total mucocyte volumes prior to and pursuant to this size. These findings are relevant in the context of mortalities in naturally-recruiting populations, as well as in the context of oyster-rearing operations, where juvenile mortality may be severe (Lacoste et al. 2001; Huvet et al. 2004; Samain et al. 2004; Dégremont et al. 2005).

The results of the present study strengthen the developmental data previously obtained for the *C. gigas* gill (Cannuel and Beninger 2006), and underscore the relevance of using different techniques to investigate particle processing in oysters and other bivalves. Experimental work on the different aspects of particle processing in the developmental stages now documented for *C. gigas* would be most appropriate at this point.

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