RESEARCH ARTICLE

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Gill development, functional and evolutionary implications in the Pacific oyster *Crassostrea gigas* (Bivalvia: Ostreidae)

Abstract Development of the *Crassostrea gigas* gill was studied in order to better understand the feeding biology of early life stages, identify potentially critical developmental stages which may influence rearing success or recruitment to wild populations, and shed light on the evolution of the basic bivalve gill types. Larvae and juveniles were reared in an experimental hatchery, and larger specimens were obtained from a commercial hatchery. Specimens were relaxed, fixed, dried, and observed using scanning electron microscopy (SEM). The right and left gills developed symmetrically, via a "cavitation–extension" process from the gill buds. The inner demibranchs developed first (V-stage, 0.29–2.70 mm), in a sequential postero-anterior series of homorhabdic filaments. The outer demibranchs developed later (W-stage, from 2.70 mm), also as homorhabdic filaments, synchronously along the gill axis. The principal filaments (PF) developed from the progressive fusion of three ordinary filaments (OF), at a size of 7.50 mm, and the consequent plication was accentuated by the formation of extensive tissue junctions. Effective filament number (number of descending and ascending filaments) showed a marked discontinuity at the transition from the V- to the W- stage of the gill. Filament ciliation showed several important changes: establishment of OF ciliation in the homorhabdic condition (2.70 mm), ciliary de-differentiation of the PF in the heterorhabdic condition (7.50 mm), and establishment of a latero-frontal cirri length gradient from the plical crest to the PF base. Reversal of direction of ciliary beat is also necessary prior to adult functioning of the PF. Three major transitions were identified in *C. gigas* gill development, each potentially important in rearing success or wild population recruitment: (1) transition from velum to gill at settlement, (2) transition from a V- to a W-shaped gill (2.70 mm), and (3) transition from the homorhabdic to the heterorhabdic condition (7.50 mm). Complete gill development was much more prolonged than in species previously studied. The major ontogenetic differences between the *C. gigas* heterorhabdic pseudolamellibranch gill and the pectinid heterorhabdic filibranch gill suggest that the heterorhabdic condition evolved independently in these two bivalve families.

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Introduction

Many benthic marine invertebrates possess two distinct stages in their life cycles: a pelagic larval stage, and a juvenile to adult benthic stage. In natural and cultured populations, the transition from pelagic to benthic life, and concomitant metamorphosis, is often associated with high mortalities (Quayle 1952; Rumrill 1990; Roegner 1991; Baker and Mann 1994a; Roegner and Mann 1995; Gosselin and Qian 1996, 1997; Gosling 2003a). The process of settlement and subsequent recruitment determine benthic population structure (Gosselin and Qian 1997; Hunt and Scheibling 1997), as well as temporal population continuity in their habitats and colonization of new habitats by means of larval dispersal (Pechenik 1999). Both abiotic and biotic factors, or interactions of both, may be responsible for high mortalities at settlement, and subsequent poor recruitment. Biotic factors known to affect these processes in benthic marine invertebrates, and in bivalves in particular, include predation (Krantz and Chamberlin 1978; Ventilla 1984; Gosselin and Qian 1997; Masski and Guillou 1999; Newell et al. 2000), competition for space and/or food (Osman et al. 1989; Zajac et al. 1989; Hunt and Scheibling 1997), diseases (Ventilla 1984; Bricelj et al. 1992; Gosselin and Qian 1997; Gosling 2003b), and energy depletion (Ó Foighil et al. 1990; Whyte et al.

1992; Gosselin and Qian 1997; Hunt and Scheibling 1997) can influence settlement success and subsequent juvenile mortality. Another biotic factor which may influence natural mortality is abnormal development (Rumrill 1990; Gosselin and Qian 1997; Hunt and Scheibling 1997) or complications during development (Roegner 1991). Even normal development is known to be a potential weak link in the growth and survival of early stages (Ó Foighil et al. 1990; Kingzett 1993; Baker and Mann 1994a; Beninger et al. 1994; Veniot et al. 2003).

Successful marine fisheries and culture operations rely in large part on successful settlement, early benthic growth/development, and subsequent recruitment to exploited populations. Oysters are the leading bivalve aquaculture species worldwide, with over 95% of landings from culture operations; of this, approximately one-third is due to a single species, *Crassostrea gigas* (data from Gosling 2003c). Despite the economic importance of this species, very little is known concerning its development, and in particular the development of the primary particle processing structure, the pseudolamellibranch gill. Most of the previous studies have provided summary descriptions of the more obvious developmental events in the genus *Ostrea* (Lacaze-Duthiers 1856; Jackson 1888, 1890; Yonge 1926; Cole 1937, 1938; Raven 1958; Walne 1974). More recently, Waller (1981) documented the formation of a gill epithelial bridge prior to the differentiation of the first gill buds in the veliger of *Ostrea edulis*. A more complete study of gill development has been performed for *Ostrea chilensis* (Chaparro et al. 2001); however, it should be noted that, unlike the genus *Crassostrea*, all members of the genus *Ostrea* brood their larvae on the gills. Moreover, key aspects of its gill development such as principal filament differentiation and ciliation, as well as plication and complete interfilamentar junctions were not reported in this study.

The present work is a detailed account of gill development in the larva, juvenile, and early adult of *C. gigas* with a view to enhancing our understanding of the biotic processes affecting recruitment into natural or cultured populations.

Study of the bivalve gill is proving increasingly useful as a phylogenetic and taxonomic tool (Beninger and Dufour 2000; Beninger et al. 2003; Neumann and Kappes 2003; Dufour et al. 2005). A secondary objective of this work is therefore the application of gill development data to the interpretation of the evolutionary affinities of the Ostreidae and the evolutionary trajectories of the major gill types within the Bivalvia.

Materials and methods

In view of the prolific synonymy of the different developmental stages from egg to adult, and the extended duration of the sub-adult phase, the following terminology was adopted in the present study: "larva" refers to pelagic stages, while "juvenile" refers to settled benthic stages up to the acquisition of a fully developed "adult" gill.

Larval and juvenile rearings

Fertilizations, larval and juvenile cultures of *C. gigas* were conducted at the Argenton experimental hatchery (IFREMER—Finistère, France; for details see Cannuel and Beninger 2005). Briefly, oocytes and spermatozoa were obtained in April 2002 by gonad stripping of adult oysters previously conditioned during 6 weeks. Larval cultures were performed at an initial density of 30–35 larvae mL^{-1}, in 150 L rearing tanks of aerated 1-μm filtered sea water (FSW). At day 2, larval concentrations were reduced to 2 larvae mL^{-1}. Larvae were fed *ad libidum* with a 1:1:1 mixture of *Pavlova lutheri*, *Isochrysis galbana* (clone T-Iso) and *Chaetoceros calcitrans* (PTC—diet, Robert and Gérard 1999; Robert et al. 2001). Initial culture temperature was 22°C, gradually raised to 24–25°C on day 6, and maintained at this temperature to settlement (Robert and Gérard 1999).

At day 20, when the majority of the larvae presented an eyespot and swimming–crawling behaviour, they were transferred to rectangular PVC sifters of 140 μm diagonal mesh placed in raceways and filled with finely crushed oyster shells, at a density of 60,000 larvae per sifter, for settlement, metamorphosis and juvenile rearing. Juveniles were fed continuously with PTC diet in 5-μm FSW. Raceways and sifters were washed first 1 week after settlement and then every 2–3 days. Juvenile rearing was stopped at day 36 (2.70 mm in shell size, Table 1). Specimens were regularly sampled during larval and juvenile rearings (1–3 day intervals).

Juvenile and adult collection

C. gigas juveniles larger than 2.70 mm were obtained from the Vendée Naissain company (Vendée, France) in September and December 2003, where they were fed with *Skeletonema costatum*. Four stages were studied: 2.85, 7.50, 10.06 and 23.83 mm, corresponding to mean shell lengths of four commercial sifter-sizes (Table 1).

Adult *C. gigas* were collected in the field (Baie de Bourgneuf, France, 47°5′ S, 2°5′ W) in May 2004, narcotized and fixed according to the protocol described below.

Scanning electron microscopy (SEM) preparation

Specimens were first narcotized in ascending concentrations of MgCl$_2$ up to 7.5% in narcotization beakers (Veniot et al. 2003) to ensure valve unlocking, tissue penetration, and then optimal fixation of non-contracted structures. Specimens were then fixed in slightly hyperosmotic 2.5% glutaraldehyde in 0.2 M buffered

sodium cacodylate for a minimum of 48 h and stored at 4°C prior to dehydration (Beninger et al. 1995). Samples were dehydrated in an ascending ethanol gradient after removal of the fixative with several washes with 0.2 M phosphate buffer, pH 7.2. They were then immersed in 100% hexamethyldisilazane (HMDS) for a night (Nation 1983; Braet et al. 1997; Heraty and Hawks 1998; Hochberg and Litvaitis 2000). Samples were removed and excess HMDS was evaporated under a fume hood. Smaller samples (larvae and juveniles up to 7.50 mm) were then mounted on stubs using double-sided adhesive tape, and a fine tungsten needle was used to remove one valve, allowing visualization of internal organs. For larger specimens (7.50 mm to adult size) or detailed observation of organs, soft parts were first removed from the shell and dissected, when necessary, before mounting on a SEM stub, allowing optimal coating and subsequent visualization. Samples were then sputter-coated with gold and observed with a scanning electron microscope (JEOL 6400).

After preliminary observations encompassing most sampling dates, detailed observations were performed for dates which represented the major developmental events (Table 1).

Results

The nomenclature used herein is the standard vocabulary of bivalve gill anatomy, with one exception: we consider the trough-shaped principal filament (PF) to consist of the two lateral walls and the trough bottom, corresponding in fact to the ontogenetic fusion of three ordinary filaments (OF) (see below). The term "transitional filament", used only in literature dealing with the Ostreidae (Galstoff 1964; Eble and Scro 1996), is thus not used here to designate the principal filament lateral wall. This conforms to the terminology already in use for the principal filament of the Pectinidae.

Structure dimensions, including ciliary lengths reported in the present work, are those obtained directly from the micrographs; it should be noted that shrinkage due to specimen preparation is uniform (Gusnard and Kirshner 1977), and estimated at 15–20% in bivalve ciliated epithelia (Beninger et al. 1999).

Overview of major developmental events

Major changes in the gill anatomy of *C. gigas* were observed over the wide shell size range explored in this

Table 1 *Crassostrea gigas*: summary of the developmental events occurring at identified sizes and ages, from larva to adult

Mean shell size (mm)	n	± SD (mm)	Age	Metamorphosis and filament ontogeny	Gill filament ciliation	Gill junctions
0.29	6	0.03	15 days	Velum fully developed Foot sparsely ciliated ~5 unciliated gill buds (ID, inner demibranchs) *Behaviour*: swimming larvae	Absent	Absent
0.33	7	0.02	20 days	Velum still fully developed Foot abundantly ciliated ~8–10 gill filaments and buds (ID), "cavitation/extension" process *Behaviour*: substrate search ("swimming–crawling")	Lateral and ventral cilia in the most anterior gill filaments	
0.42	9	0.04	22 days	Velum and foot totally regressed ~10–12 gill filaments (ID) *Behaviour*: beginning of irreversible fixed condition	Lateral cilia Median coarse and lateral fine frontal cilia	Ventral ciliary junctions
1.30	5	0.13	29 days	~20–24 gill filaments (ID)	Densification of gill ciliation	Interfilamentar and interlamellar junctions
2.70	5	0.57	36 days	~40–50 gill filaments (ID) Rapid development of the outer demibranchs (OD)	Differentiation of latero-frontal cirri	
2.85	3	0.11	10 weeks	~65 gill filaments Ventral tract plate		
7.50	5	0.50	13 weeks	~165 gill filaments Beginning of principal filament(PF) differentiation (ID and OD) Formation of the ventral groove (ID)	De-differentiation of frontal ciliation in the PF	Interfilamentar fusion
10.06	30	1.16	16 weeks	~215 gill filaments Progressive gill plication	Densification of gill ciliation	
23.83	30	2.62	22 weeks	Increased gill plication		Intraplical junctions
92.33	3	8.62	–	Gill fully developed		

Reliability of age as a development marker decreases throughout development

study (0.29–92.33 mm). From gill organogenesis initiation to complete adult gill structure, several successive stages were distinguished:

1. Development of the two opposing inner demibranchs (ID) with elongation and addition of new gill filaments from the fused posterior mantle margin (the "budding zone"), in a "cavitation–extension" process (described below), resulting in the simultaneous formation of both ascending and descending filaments of lamellae of the homorhabdic unplicate V-shaped gills (0.29–0.42 mm); this initiation of gill development was followed by progressive onset of gill junctions: first ciliary junctions and then tissue junctions (0.42–1.30 mm).
2. Delayed differentiation of the outer demibranchs (OD) and multiplication of OF (2.70 mm), resulting in the doubling of the particle capture-transport and respiratory surface; acquisition of the characteristic W-shaped gills (still homorhabdic and unplicate), formed by flat lamellae.
3. Differentiation of the PF together with interfilamentar fusion (7.50 mm), corresponding to the acquisition of the heterorhabdic condition (heterorhabdic lightly plicate gill).
4. Enlargement of the PF, gill plication (deeply plicate heterorhabdic gill) with increasing gill fusion complexity, and then acquisition of the typical "adult" gill of *C. gigas* composed of fully plicate lamellae (7.50–23.83 mm).

Detailed chronology of developmental events

0.29 mm/15 days

Young pediveliger larvae (0.29 mm shell size, 15 days) exhibited ~5 unciliated solid gill buds on each side of the budding zone (Fig. 1a, b, c; Fig. 2), the most recently differentiated gill bud measuring ~15 μm. The right and left gill buds were conjoined by mantle fusion. The velum was fully developed at this stage, and the sparsely ciliated foot extended ventrally between the two valves of the prodissoconch II shell (Fig. 1b).

0.33 mm/20 days

In 0.33 mm/20 day (competent for metamorphosis) late pediveliger larvae, ~8–10 gill filament rudiments, arising from the gill buds, were observed on each side of the foot (Fig. 1d) corresponding to the future left and right gill, the most anterior filament measuring ~30 μm in length. Both ascending and descending lamellae of the ID developed simultaneously in a "cavitation–extension" process (Fig. 2). A perforation in the gill filament rudiment was followed by progressive ventralward elongation of the ventralmost region of the filament rudiment, resulting in the simultaneous genesis of the highly-constrained suprabranchial cavity, the infrabranchial cavity, and the ascending and descending filaments—hence the term "cavitation–extension". Lateral (~5 μm long) and ventral cilia (~3–4 μm long) first developed in the anterior gill filaments, while the most posterior (more recently differentiated) filaments arising from the budding zone were unciliated (Fig. 1d). In the two most anterior gill filaments, short frontal cilia (range: 2.5–7 μm) began to develop. The velum was still well developed (not visible on micrographs), and the long foot was abundantly ciliated.

0.42 mm/22 days

The 0.42 mm/22 day juveniles underwent metamorphosis, as attested by the totally regressed velum and foot (Fig. 1e). The cavitation–extension process was quite evident (Figs. 1e, 2), and juveniles at this stage exhibited 10–12 gill filaments (~60 μm long for the anteriormost filament) on both equally-developed left and right gills. This stage also corresponded to the acquisition of abundant gill ciliation (Figs. 1e, 5a). Except in the 4–5 most recently-developed gill filaments, where ciliation was absent to sparse, the frontal surface of the most anterior gill filaments was covered by several distinct ciliary types. Abundant, simple lateral cilia (~7 μm) were observed on either side of the frontal surface. On the frontal surface, a median tract of long, apparently composite cilia (~8.5 μm) and two lateral tracts of shorter simple cilia (~3.5 μm) developed (Fig. 5a). Although the frontal surfaces of the gill filaments were now abundantly ciliated, in contrast, the abfrontal surfaces were very sparsely ciliated, with a single central row of very short simple cilia (~1 μm; Fig. 5b). The anteriormost gill filaments appeared to be joined at their ventral extremity by ciliary junctions connecting two adjacent filaments (Fig. 1e). No ventral particle groove was observed at this stage, whereas the dorsal groove formed by the gill arch exhibited scattered long simple cilia (~10 μm; Fig. 1e). Labial palp primordia appeared at the anterior gill extremity.

1.30 mm/29 days

In 1.30 mm/29 day juveniles, the first organic gill junctions were observed. Previously joined by ventral ciliary junctions, the gill filament ventral extremities were now fused via ~10 μm-wide tissue junctions, covered ventrally by short simple cilia (~5 μm; Fig. 1g). No ventral particle groove was yet visible. Organic interfilamentar junctions between adjacent filaments of the same gill lamella appeared in some individuals (Fig. 6a), but this was not yet the general case in this size-class. In abfrontal view, organic interlamellar junctions were clearly visible (Fig. 1f); the adjacent interlamellar junctions were separated by ~5 gill filaments. The abfrontal surfaces remained poorly ciliated, exhibiting a single row of
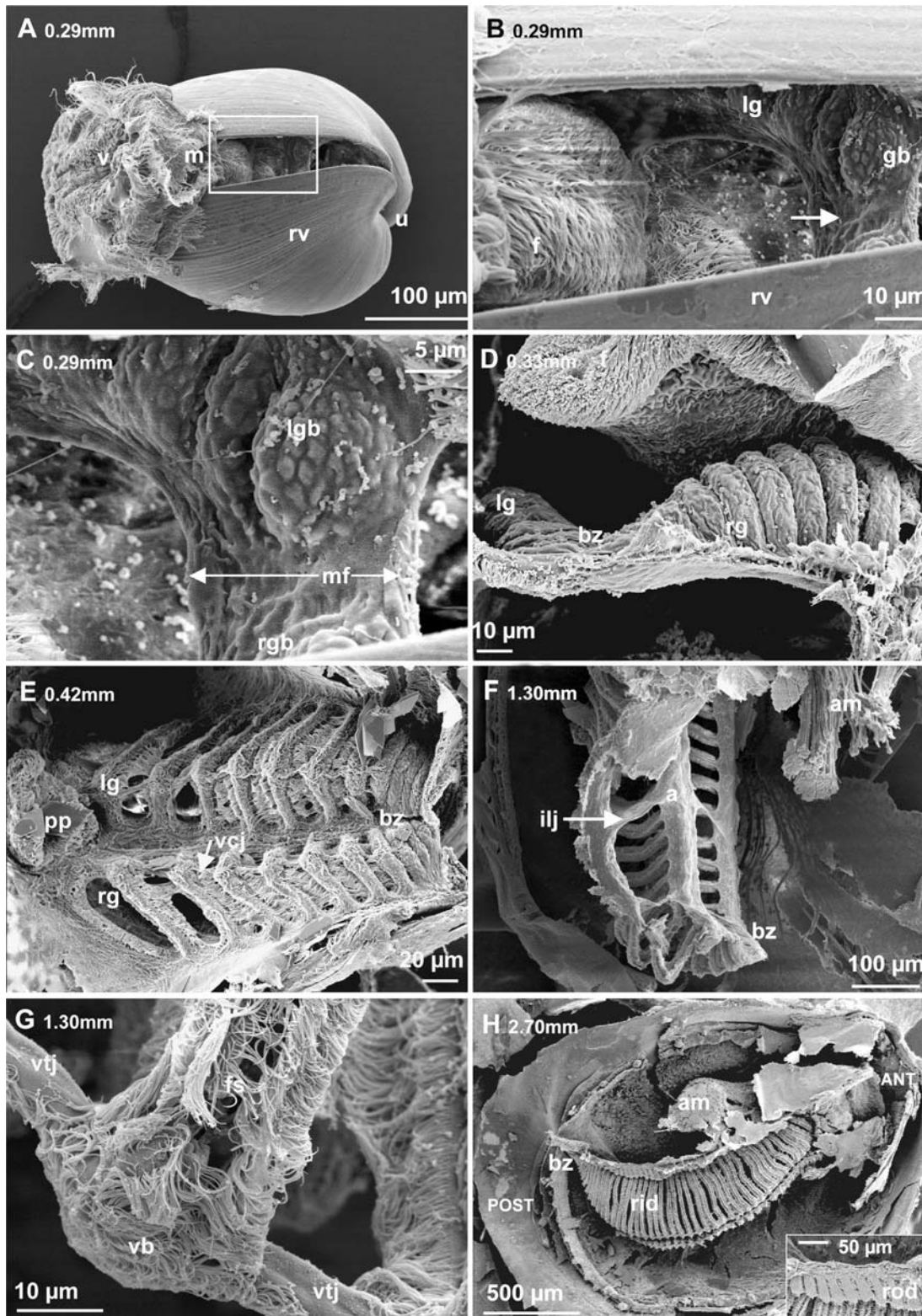
Fig. 1 *Crassostrea gigas*: gill ontogeny. **a** Ventral view of a 0.29 mm/15 day veliger larva. *m* mouth location, *rv* right valve, *u* umbo, *v* velum, *rectangle* detail in 1B. **b** Detail of **a**. *f* foot, *gb* gill bud, *lg* left gill, *arrow* gill bridge. **c** Detail of **b**: gill buds on both sides of mantle fusion. *lgb* left gill bud, *mf* mantle fusion, *rgb* right gill bud. **d** Posterior-ventral view of a 0.33 mm/20 day pediveliger larva. *bz* budding zone, *rg* right gill. **e** Ventral view of a 0.42 mm/ 22 day juvenile. *pp* labial palp primordia, *vcj* ventral ciliary junction. **f** Abfrontal view of gill of a 1.30 mm/29 day juvenile. *a* gill axis, *am* adductor muscle, *ilj* interlamellar junction. **g** Close-up of the ventral extremity of a demibranch of a 1.30 mm juvenile. *fs* frontal surface, *vb* ventral bend, *vtj* ventral tissue junction. **h** Lateral view of a 2.70 mm/36 day juvenile, *inset* detail of the developing outer demibranch. *rid* right inner demibranch, *rod* right outer demibranch, *ANT–POST* organ orientation

Fig. 2 *C. gigas:* diagram of gill ontogeny. *Dotted line* antero-posterior axis of symmetry. *al* ascending lamella, *dl* descending lamella, *id* inner demibranch, *od* outer demibranch, *vg* ventral groove, *vtp* ventral tract plate
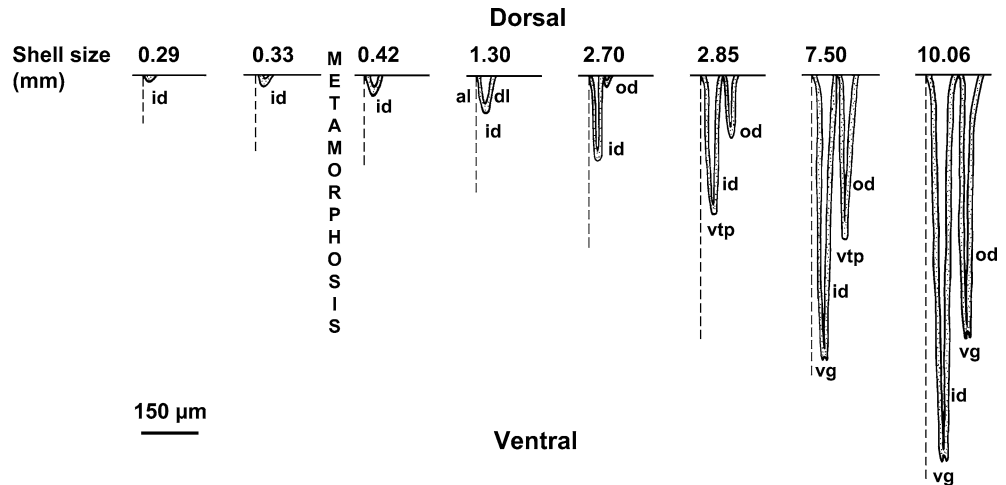
very small marginal cilia on each side of the filament ($\sim$1 μm) and a central single row of small simple cilia ($\sim$3 μm), with an interciliary distance of 2–4 μm (Fig. 5c). Gill ciliation appeared generally more dense than in 0.42 mm juveniles. 20–24 gill filaments $\sim$120 μm in length (mid-gill on an antero-posterior axis) were observed on each demibranch at this stage.

2.70 mm/36 days

In 2.70 mm/36 day juveniles, developing OD were first observed when the filaments of the ID were $\sim$300 μm in length. The OD developed in a radically different manner from that of the ID: all filaments developed simultaneously and synchronously along the gill axis, parallel to the already-developed ID (Figs. 1h, 2), via the cavitation–extension process described above. Although this marked synchrony was observed within each demibranch, development of the right and left OD was not necessarily synchronous. When the 40–50 gill filaments composing the ID were $\sim$350 μm in length, the filaments of the OD were $\sim$50 μm long. At this stage, two non-terminal interfilamentar junctions joined adjacent filaments (Fig. 6b); the number of organic junctions increased with gill filament growth. The spaces bounded by the interfilamentar junctions comprised the ostia.

A major change occurring in gill ciliation was the differentiation of latero-frontal cirri on the gill filament frontal surfaces. These consisted of groups of 4–5 apparently composite cilia ($\sim$8.5 μm) regularly spaced every 3 μm dorso-ventrally, between the simple frontal cilia and the lateral cilia ($\sim$9 μm) of the gill filaments (Fig. 5d). No ventral particle groove was yet visible (Figs. 1h, 2, 6b).

2.85 mm/10 weeks

The 2.85 mm/10 week juveniles exhibited $\sim$65 gill filaments, $\sim$460 μm in length for the ID, $\sim$210 μm in length for the OD, with 4–5 and 2 non-terminal interfilamentar junctions in the ID and the OD, respectively (Fig. 6c). Ostia closest to the dorsal particle groove were smaller than the others ($\sim$28 vs. 68 μm; Fig. 6c). The ventral particle groove began to develop in the ID at this stage, the convex ventral bend initially becoming a flat surface (Figs. 2, 3a, 6c). It was abundantly ciliated with long, apparently composite, cilia ($\sim$12–15 μm).

7.50 mm/13 weeks

In 7.50 mm/13 week juveniles, a major event in gill development occurred: the beginning of PF differentiation, conferring a three-dimensional aspect to gill lamellae, which appeared to be slightly plicate in several places (Fig. 3b). Between two plicae, frontal observation showed one enlarged OF ($\sim$20–22 μm width) surrounded by two OF ($\sim$11 μm width), progressively fusing and filling the ostia delimited by interfilamentar junctions (Figs. 3c, 4a, b). This triplet of OF composed the future PF, the enlarged OF constituting the PF base, and the two fused OF constituting the lateral walls of the PF. OF frontal ciliation showed the ciliary types previously described for 2.85 mm juveniles: lateral cilia ($\sim$9.9 μm), composite ($\sim$8.1 μm) and simple ($\sim$3.1 μm) frontal cilia, and latero-frontal cirri (groups of $\sim$4–6 cilia, $\sim$7.1 μm; Fig. 5 e). However, some de-differentiation of frontal ciliation occurred in the PF base, as shown in Fig. 3c: composite frontal cilia could no longer be distinguished, and the frontal surface appeared to be covered by simple cilia ($\sim$7–8 μm). It is probable that PF differentiation occurred at the sites of interlamellar junctions, because these mainly corresponded to the plical troughs (Fig. 6d, e).

The second major event occurring at this developmental stage was the progressive invagination of the ventral particle groove (Figs. 2, 3b) at the ventral extremity of the ID. The formation of the OD ventral groove was not contemporaneous with that of the ID; at this stage, the future OD groove became a flat surface,
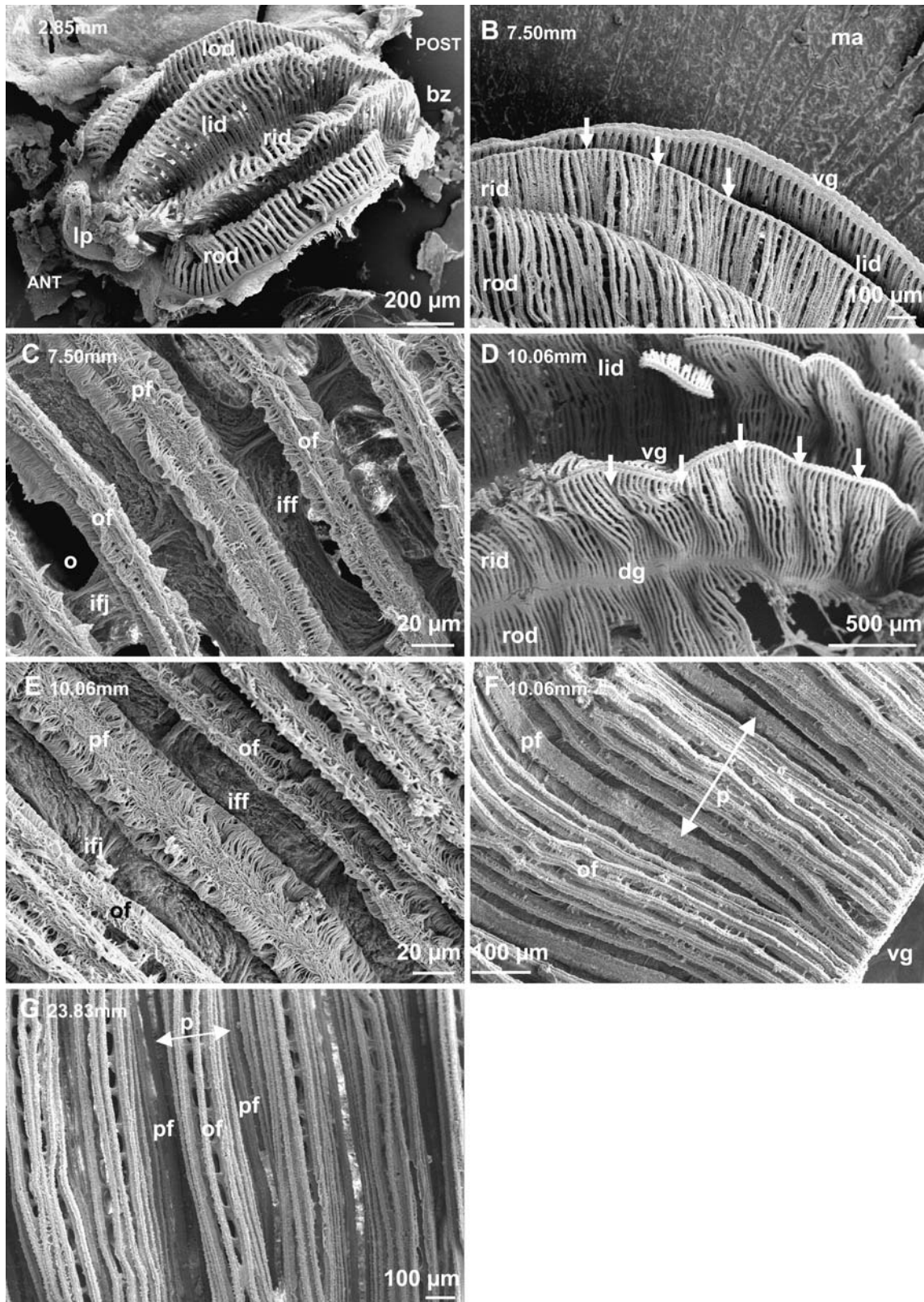
Fig. 3 *C. gigas:* principal filament differentiation. **a** Ventral view of a 2.85 mm/10 week juvenile. *bz* budding zone, *lid* left inner demibranch, *lod* left outer demibranch, *lp* labial palps, *rid* right inner demibranch, *rod* right outer demibranch, *ANT–POST* organ orientation. **b** Lateral view of a 7.50 mm/13 week juvenile. *ma* mantle, *vg* ventral groove, *arrows* location of principal filament differentiation (plication initiation). **c** Frontal view of a gill lamella of a 7.50 mm juvenile. *iff* interfilamentar fusion, *ifj* interfilamentar junction, *o* ostium, *of* ordinary filament, *pf* principal filament (differentiating). **d** Ventral view of a 10.06 mm/16 week juvenile. *dg* dorsal groove, *arrows* location of the differentiating principal filaments. **e** Frontal view of a gill lamella of a 10.06 mm juvenile. **f** Frontal view of the ventral extremity of a gill lamella of a 10.06 mm juvenile. *p* plica. **g** Frontal view of gill plicae of a 23.83 mm/22 week juvenile
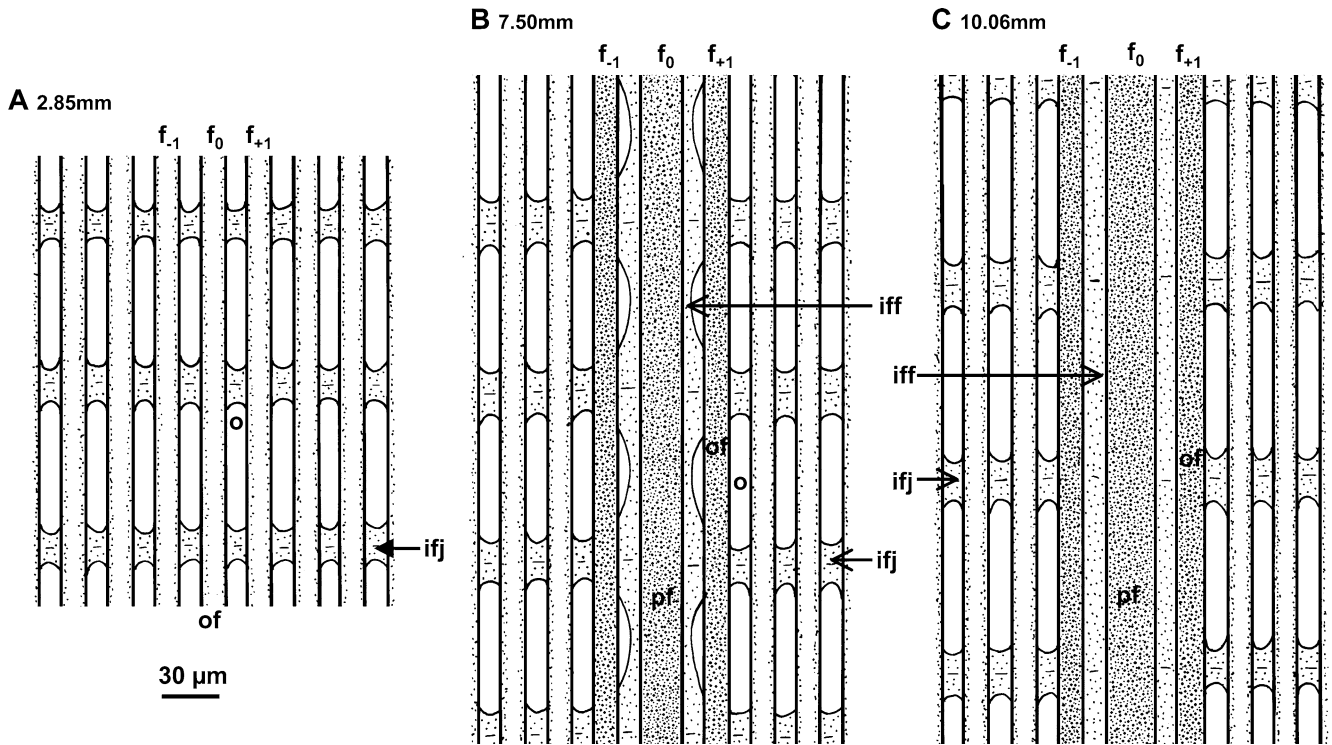
Fig. 4 *C. gigas*: diagram of principal filament differentiation. Frontal views of gill lamellae for each developmental stage. **a** A 2.85 mm/10 week juvenile. *f* filament, *ifj* interfilamentar junction, *o* ostium, *of* ordinary filament. **b** A 7.50 mm/13 week juvenile. *iff* interfilamentar fusion, *pf* differentiating principal filament. **c** A 10.06 mm/16 week juvenile

representing the first step in groove formation (Fig. 2). Both ventral particle grooves were abundantly ciliated with groups of apparently composite cilia (~12–20 μm).

10.06 mm/16 weeks

In 10.06 mm/16 week juveniles, gill plication continued as shown in Fig. 3d. PF bases were now ~24 μm in width at the dorso-ventral midpoint of the gill lamellae, while no larger than an OF at the ventral extremity of gill lamellae (~12 μm), hence allowing plication (Fig. 3f). OF width remained constant since the preceding developmental stage. Interfilamentar fusion of the three filaments comprising each PF seemed to be nearly complete in frontal view (Figs. 3e, 4c). Gill frontal ciliation increased in density in the OF (Fig. 5f), with lateral cilia (~10 μm), composite (~8.2 μm) and simple (~3.1 μm) frontal cilia, and latero-frontal cirri (~8.1 μm). Abfrontal ciliation remained very sparse, with abfrontal cilia and marginal cilia (Fig. 5g) as described for 1.30 mm individuals. The PF base frontal ciliation differed clearly from that of OF, with complete de-differentiation of composite and simple frontal cilia (Fig. 5h), and was composed of simple cilia (~8–10 μm). Latero-frontal cirri were still visible on each side of the PF base frontal surface (Fig. 5h). Small (~40–50 μm) and very small (~10–20 μm) ostia bordered the dorsal food groove, while the ostia between the PF and the first OF measured

~40 μm, and the remaining ostia were ~80 μm in length and ~30 μm in width (Fig. 6e). Gill filaments of the ID were joined to each other by 12–14 rows of interfilamentar junctions (Fig. 6e).

23.83 mm/22 weeks

Gill architecture increased in complexity in 23.83 mm/22 week juveniles. Progressive gill plication was followed by the appearance of intraplical junctions joining the abfrontal surfaces of two adjacent PF (Fig. 6f, g), completing the three-dimensional structure of the gills (Fig. 3g). The ostia between the PF and the first OF measured ~50 μm at this stage.

92.33 mm/Adult

In adult oysters, gill plicae were more pronounced than in 23.83 mm juveniles, preventing frontal observation of the PF in intact dissected gills (Fig. 7a). Intraplical junctions were more developed than hitherto, but no additional complexity in gill junctions was observed (Fig. 7d). These developments marked the completion of gill ontogeny. OF ciliation was composed of composite (~10–15 μm) and simple (~5 μm) frontal cilia, latero-frontal cirri (~9–10 μm) and lateral cilia (~15 μm) (Fig. 7b). PF base frontal ciliation, observed in fractured gills, was made up of long simple frontal cilia (~10–
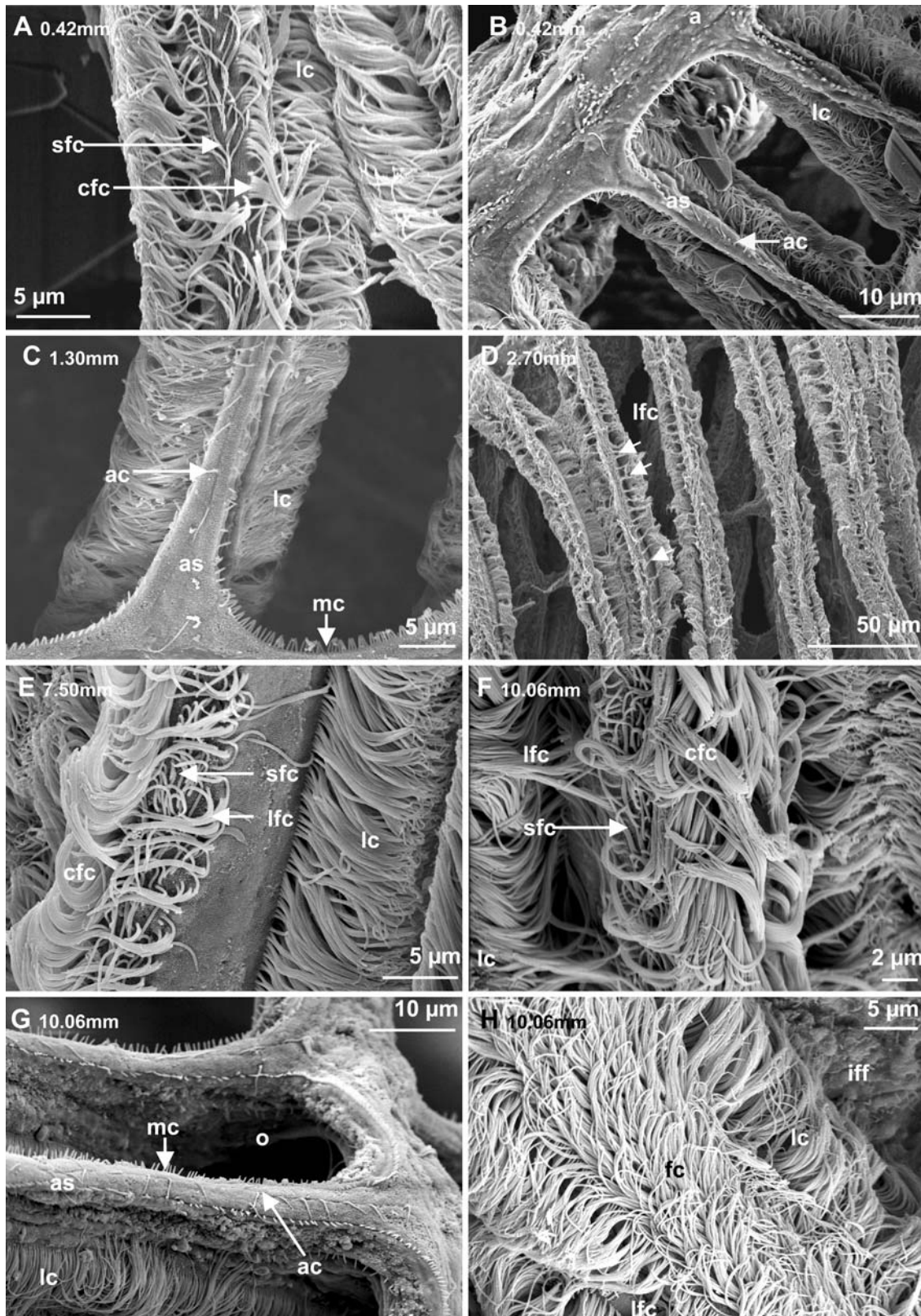
Fig. 5 *C. gigas*: gill ciliation. **a** Frontal view of a gill filament of a 0.42 mm/22 day juvenile. *cfc* composite frontal cilia, *lc* lateral cilia, *sfc* simple frontal cilia. **b** Abfrontal view of a gill filament of a 0.42 mm juvenile. *a* gill axis, *ac* abfrontal cilia, *as* abfrontal surface. **c** Abfrontal view of a gill filament of a 1.30 mm/29 day juvenile. *mc* marginal cilia. **d** Frontal view of gill filaments of a 2.70 mm/36 day juvenile. *lfc* latero-frontal cirri. **e** Latero-frontal view of a gill ordinary filament of a 7.50 mm/13 week juvenile. **f** Frontal view of a gill ordinary filament of a 10.06 mm/16 week juvenile. **g** Latero-abfrontal view of ordinary gill filaments of a 10.06 mm juvenile. *o* ostium. **h** Frontal view of a gill principal filament of a 10.06 mm juvenile. *fc* frontal cilia, *iff* interfilamentar fusion
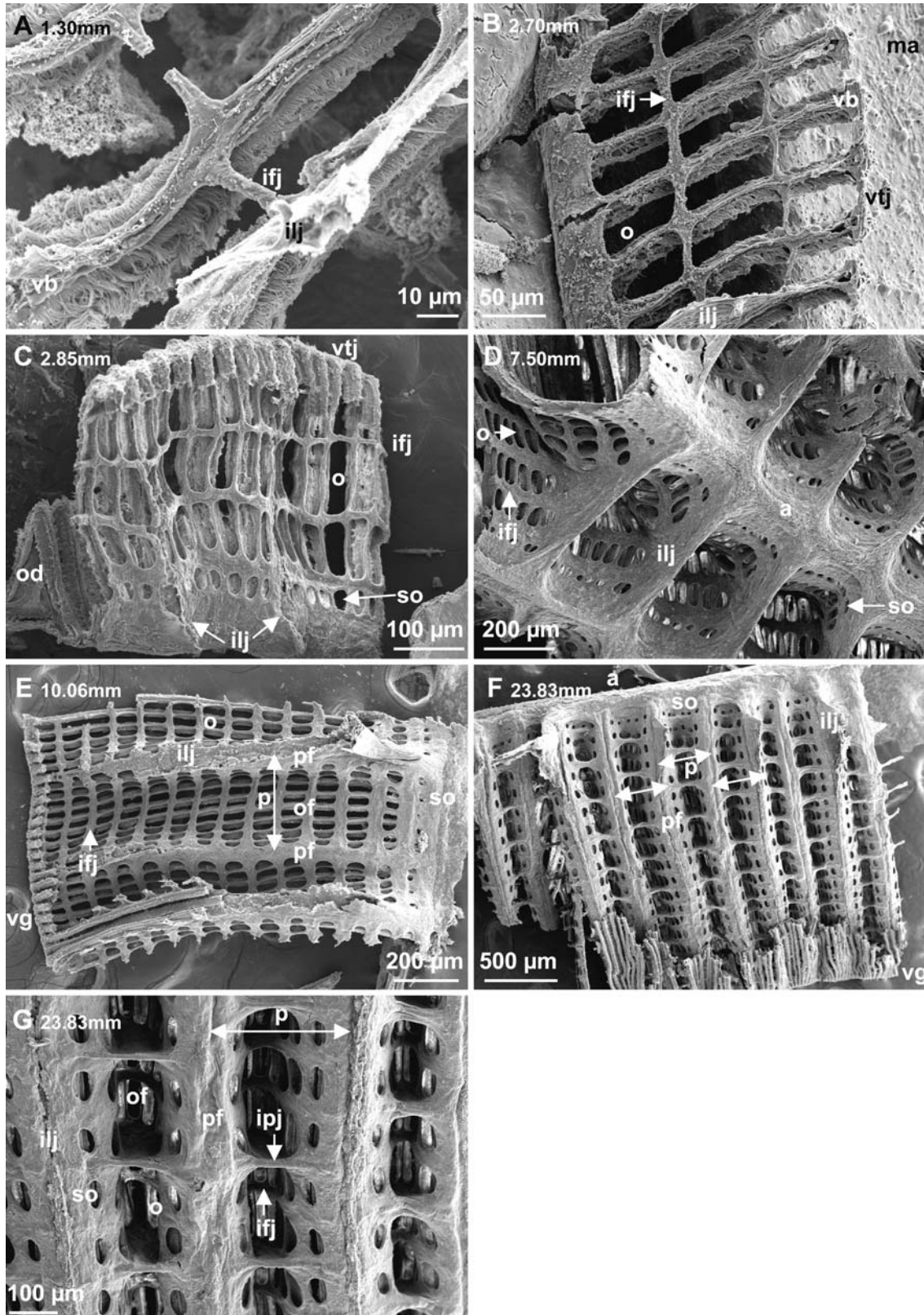
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Fig. 6 *C. gigas*: ontogeny of gill junctions. **a** Abfrontal view of gill filaments of a 1.30 mm/29 day juvenile. *ifj* interfilamentar junction (sectioned), *ilj* interlamellar junction (sectioned), *vb* ventral bend. **b** Abfrontal view of a gill lamella of a 2.70 mm/36 day juvenile. *ma* mantle, *o* ostium, *vtj* ventral tissue junction. **c** Abfrontal view of a gill lamella of a 2.85 mm/10 week juvenile (interlamellar junctions sectioned). *od* outer demibranch, *so*: small ostium. **d** Dorsal view of

the abfrontal surface of the gills of a dissected 7.50 mm/13 week juvenile. *a* gill axis. **e** Abfrontal view of a gill lamella of a 10.06 mm/16 week juvenile (interlamellar junctions sectioned). *of* ordinary filament, *p* plica, *pf* principal filament, *vg* ventral groove. **f** Abfrontal view of a gill lamella of a 23.83 mm/22 week juvenile (interlamellar junctions sectioned). **g** Detail of **f**. *ipj* intraplical junction
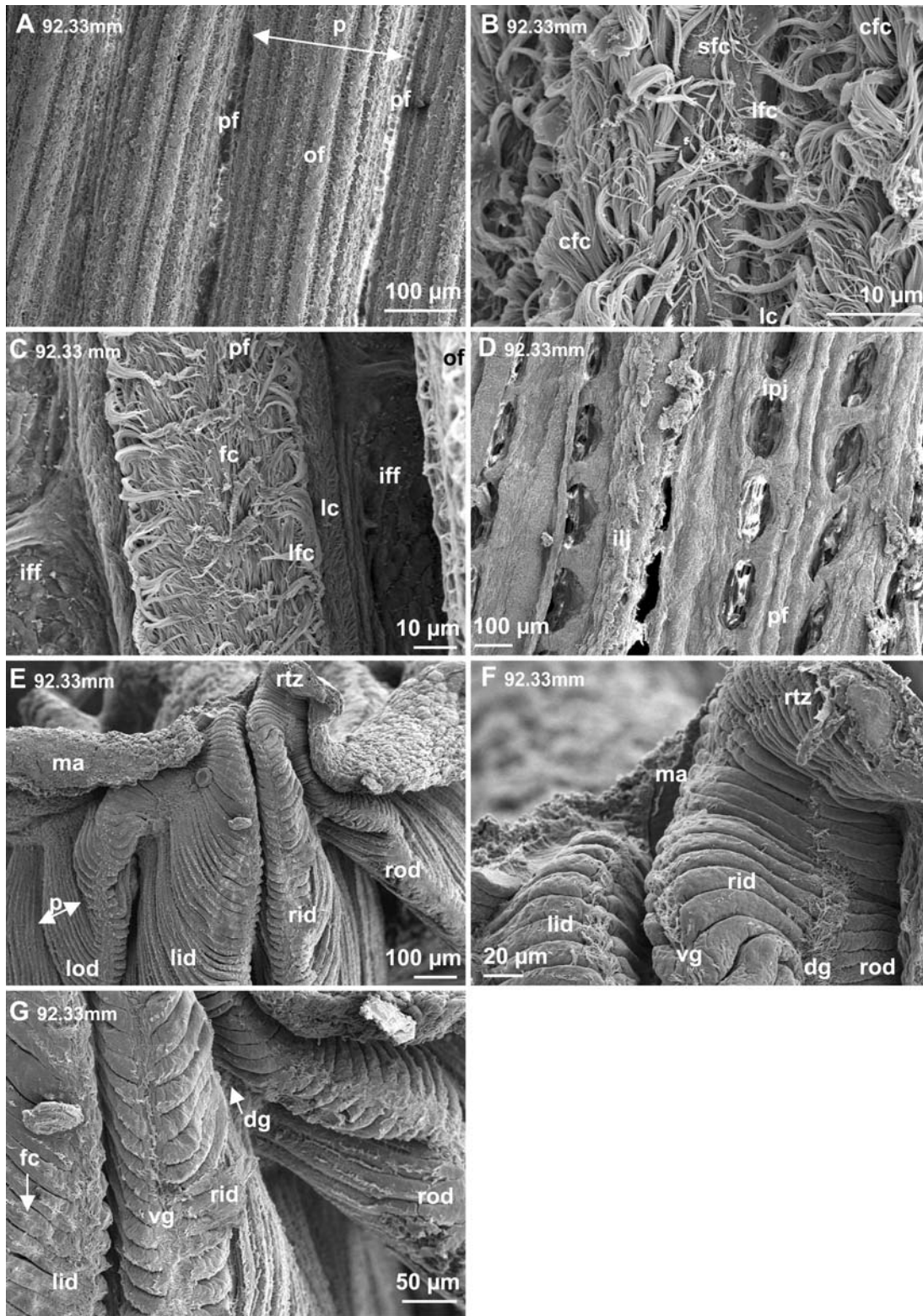
Fig. 7 *C. gigas*: adult gill. **a** Frontal view of gill plicae. *of* ordinary filament. *p* plica, *pf* principal filament. **b** Latero-frontal view of an ordinary filament. *cfc* composite frontal cilia, *lc*: lateral cilia, *lfc* latero-frontal cirri, *sfc* simple frontal cilia. **c** Frontal view of a principal filament. *fc* frontal cilia, *iff* interfilamentar fusion. **d** Abfrontal view of a gill lamella (interlamellar junctions sectioned). *ilj* interlamellar junction (sectioned), *ipj* intraplical junction. **e** Ventral view of the gill proliferation/differentiation zone. *lid* left inner demibranch, *lod* left outer demibranch, *ma* mantle, *p* plica, *rid* right inner demibranch, *rod* right outer demibranch, *rtz* right transition zone. **f** Detail of the gill proliferation zone presented in **e**. *dg* dorsal groove, *vg* ventral groove. **g** Detail of the differentiation zone of the gill presented in **e**. *fc* frontal cilia

Fig. 8 *C. gigas*: Linear regressions of shell size (mm) and effective filament number, and summary of gill major developmental events and associated potential critical stages. *Open star symbol* potential critical stage
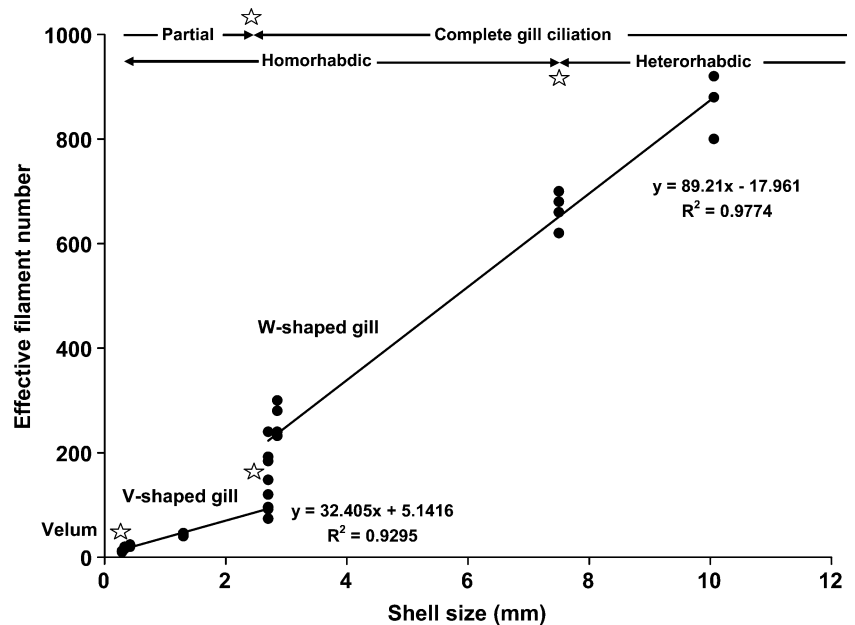
15 μm), latero-frontal cirri (~17–18 μm) and lateral cilia (~12–13 μm). PF lateral wall ciliation was composed of composite (~10–15 μm) and simple (~5 μm) frontal cilia, latero-frontal cirri (~13–14 μm), and lateral cilia (~15 μm) (Fig. 7c). PF abfrontal ciliation was composed of groups of irregularly distributed cilia (~5–10 μm), except at the sites of interlamellar junctions, where tissue fusion obviated the presence of cilia (Fig. 7d).

In contrast to the preceding developmental stages, the gill buds did not directly give rise to elongating filaments. Rather, they first expanded to form a transition zone of conjoined bar-shaped left and right gill rudiments (Fig. 7e, f). Gill elongation in the adult budding zone also differed from that observed in young juveniles. A single gill bar in the transition zone appeared to give rise to both ID and OD simultaneously (Fig. 7e, g), rather than to the ID only, as in the gill buds of young juveniles (see above). Ventral and dorsal groove ciliary tracts differentiated rapidly in the elongating filaments of adults (Fig. 7f), while the gill filament frontal surfaces initially remained unciliated. Frontal cilia then appeared, but because of the compactness of gill filaments, lateral cilia were not observed (Fig. 7g). PF differentiation also occurred rapidly, as evidenced by the two plicae observed in Fig. 7e.

Filament number and shell size

Counts of gill filaments on SEM micrographs were related to shell size over the range 0.29–10.06 mm (the gills of larger specimens tended to curve out of the plane of sight in the SEM preparations). Since gill development was symmetric, the total number of gill filaments was equal to twice the number of ID for the V-stage, and

four times the number of the ID for the W-stage (effective filament number). Separate regressions were therefore carried out for each of these stages. The results show a marked discontinuity at the transition from the V- to the W-stage, at a size of 2.70 mm (Fig. 8).

Discussion

Initial development, inner and outer demibranch formation

Gill development in *C. gigas* is here shown to be of the papillary type, as reported for a number of taxonomically-diverse genera, possessing all of the four basic gill types: *Mytilus*, *Modiolus*, *Anomia* and *Arca* (homorhabdic filibranch—Rice 1908; Raven 1958; Bayne 1971), *Pecten* and *Placopecten* (heterorhabdic filibranch—Raven 1958; Beninger et al. 1994; Veniot et al. 2003), *Ostrea edulis* (brooding heterorhabdic pseudolamellibranch—Waller 1981; Moor 1983), *Dreissenia* (non-brooding freshwater eulamellibranch—Raven 1958), and various Unionidae (brooding freshwater eulamellibranch—Raven 1958). The "cavitation–extension" mode of papillary extension to filaments, and subsequent differentiation to ID and OD, observed in the present study for *C. gigas*, is the first report of such a process in heterorhabdic bivalve gill development. Although they did not examine any heterorhabdic species, Neumann and Kappes (2003) proposed a similar mode of elongation–differentiation to be common to all bivalves. However, all three studies of the heterorhabdic filibranch gill of the Pectinidae show a distinct "elongation–reflection" mode of elongation–differentiation, in which the descending filament of the inner demibranch (ID) first elongated to form a "gill basket" with

the corresponding opposite ID, and then reflected abfrontally to form the ascending filament and achieve the 'V' shape of the ID (Kingzett 1993; Beninger et al. 1994; Veniot et al. 2003). This obviously does not exclude further filament growth, since large adults have longer gill filaments than smaller individuals, and cambial zones are distributed throughout the length of the pectinid gill filaments (Leibson and Movchan 1975).

In the pseudolamellibranch gill of *C. gigas*, both the descending and the ascending filaments of the ID formed simultaneously by "cavitation–extension", and no temporary gill basket was formed. It is likely that this is a general feature of the Ostreidae; although Chaparro et al. (2001) indicated that filament extension occurred via "reflection" in *Ostrea chilensis*, examination of their micrographs suggests that the ID of this species also develops in the "cavitation–extension" manner. This constitutes a major developmental difference between the Pectinidae and the Ostreidae for a fundamental biological character. The evolutionary implications of this will be considered below.

The mode of formation of the outer demibranch (OD) also contrasts markedly with that previously reported in the Pectinidae, in which the OD formed in essentially the same, albeit accelerated, manner as the ID: sequential appearance and elongation of the descending filament, followed by reflection (more rapid for the OD than for the ID) to form the ascending filament (Beninger et al. 1994; Veniot et al. 2003). In *C. gigas*, the development of the OD differs from that of the ID, and from that of both the ID and the OD in the Pectinidae: the descending and ascending filaments are indeed formed simultaneously by "cavitation–extension", but instead of an antero-posterior sequential appearance of gill papillae, the papillae all appear simultaneously on the gill axis along the length established by the previous development of the ID. Similarly, this contrasts with the report of sequential development of the OD filaments in *Ostrea chilensis* (Chaparro et al. 2001). Within the Ostreidae, then, there appears to be differing modes of gill development, although these differences are not as pronounced as those noted for the Pectinidae and the Ostreidae. Although only one representative of each of the two ostreid genera have been studied to date, it would be interesting to determine whether the difference in OD development constitutes yet another biological distinction between the brooding genus *Ostrea* and the non-brooding genus *Crassostrea*.

Outer demibranch (OD) differentiation in *C. gigas* was delayed in relation to that of the ID, as reported for bivalves in general (Lacaze-Duthiers 1856; Jackson 1890; Quayle 1952; Raven 1958; Kingzett 1993; Beninger et al. 1994; Baker and Mann 1994a; Korniushin 1996, 1997; Chaparro et al. 2001; Neumann and Kappes 2003; Veniot et al. 2003). Although in most cases the development of the right and left OD was synchronous, in one individual (out of five observed) there was a time lag between the two, as has been reported previously (Jackson 1890).

Due to the continuous growth of gills with increasing shell size, the budding zone remains active in adults (Neumann and Kappes 2003) and appears to attain its final form and functionality after differentiation of the OD. Before development of the OD, a single bud gives rise to the inner demibranch only, whereas after full development of the OD, a single bud becomes barshaped and gives rise to both the ID and OD. As suggested by Neumann and Kappes (2003), the simultaneous appearance of OD gill buds along the gill axis appears to occur during a short developmental period (2.70 mm shell size).

Principal filament differentiation and plica establishment

The results presented herein document, for the first time, the differentiation of principal filaments (PF) in a heterorhabdic bivalve. As previous studies have either only identified the time of first appearance of the PF (Beninger et al. 1994; Veniot et al. 2003), or ended prior to PF differentiation (Chaparro et al. 2001), it is not possible to discuss this process from a comparative standpoint. However, the process documented here for *C. gigas* is interesting in itself. The formation of the PF from three OF corresponds well to the observed morphology in the adult: the two lateral walls, both rounded and ciliated, and the trough base, also rounded and ciliated (Beninger and Dufour 1996). The formation of the PF from OF's also demonstrates convincingly the previously-supposed apomorphic relationship of the heterorhabdic gill to the plesiomorphic homorhadbic filibranch gill (Beninger and Dufour 2000). Given the relative regularity of PF's (10–18 OF per plica, PF walls excluded), the determinism of differentiation is an interesting subject for future research.

Principal filament (PF) differentiation in *C. gigas* occurs much later than in the pectinid species studied to date: 7.50 mm, compared to 4 mm in *Pecten maximus*, and 3.3–5.0 mm in *Placopecten magellanicus* (Beninger et al. 1994; Veniot et al. 2003). The time lag before appearance of PF in *C. gigas* corresponds to 91 days in the conditions of the present study; for comparison, the previous study of *Ostrea chilensis* was terminated without any sign of the PF after 86 days of development (Chaparro et al. 2001). This may be contrasted with the time lag of 56–58 days in *Pecten maximus* (Beninger et al. 1994).

Notwithstanding the slight undulation seen in eulamellibranch gills due to the presence of interlamellar junctions (Ridewood 1903; Dufour and Beninger 2001), the deep plication of the pseudolamellibranch oyster gill is a consequence of PF differentiation and formation of interlamellar junctions, interfilamentar and intraplical fusion. The latter characteristic, documented in detail in the present study, is, among the heterorhabdic bivalves, unique to the Ostreidae, and accounts for the extreme degree of plication, compared to the Pectinidae. Commensurate with this extreme degree of plication and in-

terfilamentar–intraplical fusion, is the size at which plication is complete: 7 mm in *Placopecten magellanicus* (Veniot et al. 2003), compared to 23.83 mm in *C. gigas* (present study). Full gill development is thus exceptionally slow in this oyster species, and reflects the amount of development which precedes the final form. From a functional point of view, the gill filament appears to be the basic particle processing unit in homorhabdic juvenile stages, whereas in the heterorhabdic adult stage, the gill plica appears to exert a strong influence on particle processing (Ward et al. 1998; Cognie et al. 2003). This is further supported by the gradient of mucocyte densities within a plica (Beninger and Dufour 1996; Beninger et al. 2005). This shift in gill functionality must occur gradually during development, as evidenced by the gradual plication of the gill.

Ciliation

Gill ciliation was absent or very scarce on the filaments of pediveligers, correlating to the lack of gill particle-processing functionality in larval gills. Ciliation became rapidly denser following metamorphosis, with the change from velum to gills as particle collecting structures. All four cilia types (lateral, latero-frontal, simple frontal and composite frontal) were present on the homorhabdic filaments of juveniles at 2.70 mm, and the cilia densities increased with age and size. The differentiation of the PF was accompanied by the de-differentiation of the PF base frontal cilia, which became uniformly simple.

The formation of PF from OF presents an interesting problem for ciliary function. The beat orientation of the *C. gigas* juvenile OF composite frontal cilia, prior to PF differentiation (inferred from consistent SEM micrographs), is ventralward, whereas the PF base uniform simple frontal cilia beat dorsally in the adult heterorhabdic gill. Transient ciliary beat reversal has been observed in some animal species, e.g. the avoidance response of *Paramecium*, the avoidance/feeding response in ctenophores, and the feeding responses of sabellids (Dubois et al. 2005), and appears to be mediated by instantaneous calcium transients on the ciliary membrane (Tamm and Terasaki 1994). Permanent ciliary beat reversal has not yet been documented in any bivalve gill cilia. The loss of the composite frontal cilia in the PF, which beat ventrally in the juvenile and adult OF, appears to be a precondition for permanent beat reversal, which was not yet evident in the 10.06 mm specimens which had lost their composite cilia.

The observed size gradient for latero-frontal cirri, with shortest latero-frontal cirri on the apical filaments of plicae, longest latero-frontal cirri at the PF base, and intermediate latero-frontal cirri on the PF walls, may indicate an increased role in particle capture toward the PF. It should be remembered that particles present in the PF have undergone an initial particle selection on the heterorhabdic gill (Ward et al. 1998; Beninger et al. 2004), and it is thus interesting to note the increasing size of the latero-frontal cirri, which are associated with particle capture in the homorhabdic gill (Silverman et al. 1996, 1999), toward the PF base.

The abfrontal ciliation of the OF was both sparse and regular throughout gill development, being limited to the two rows of very short simple marginal cilia, and the median row of abfrontal cilia reported here. The role (if any) of these cilia is unknown, but their limited size, number, and distribution precludes any role in water pumping, as has been suggested for the much longer and denser abfrontal ciliation of the homorhabdic filibranch *Mytilus edulis* (Jones et al. 1990, 1992). The abfrontal cilia of the *C. gigas* PF were longer and somewhat more abundant than those of the OF, but present only in isolated clumps. With the formation of the interlamellar junctions, these became even more sparse, underscoring their vestigial nature (Beninger and Dufour 2000).

Formation of the ventral particle groove

The presence of a ventral particle groove is another particularity of the adult heterorhabdic pseudolamellibranch gill (the Pectinidae do not possess such a groove). It is seen here to begin to develop quite late in *C. gigas* gill ontogeny (50 days post-settlement). The formation of the ventral particle groove in *C. gigas* corresponds precisely with the onset of PF differentiation and gill plication. That this feature should be so closely associated with the acquisition of the heterorhabdic state in *C. gigas*, whereas it is totally absent in the heterorhabdic filibranchs, constitutes another indication that heterorhabdy is a convergent evolutionary character in the Pectinidae and the Ostreidae, rather than a synapomorphy.

The lack of a true ventral groove in early developmental stages (<7.50 mm) suggests that adequate gill function is possible in early stages without such a groove (Stasek 1962); this is supported by the abundant ventral and dorsal tract ciliation, with anteriorward beating of cilia inferred from SEM micrographs (not shown here), as well as the detection of post-settlement feeding in *C. virginica* (Baker and Mann 1994b).

Evolutionary implications

The fundamental differences in heterorhabdic gill ontogeny between the Ostreidae and the Pectinidae, detailed above, lead to the conclusion that these two heterorhabdic gill types evolved completely independently from the plesiomorphic homorhabdic filibranch condition. Although it has been argued that fine details of bivalve anatomy evolve more quickly than other structures (Beninger and Dufour 2000), the ontogenetic differences reported here are extremely fundamental, and point to convergent evolution to the heterorhabdic state. This is a particularly interesting perspective, since

there are only two basic structural designs of the bivalve gill: homorhabdic and heterorhabdic. From the plesiomorphic homorhabdic filibranch condition, there thus appears to have been independent evolutionary trajectories to the heterorhabdic filibranch and pseudolamellibranch conditions, underscoring the phylogenetic distance between the Ostreidae and the Pectinidae (Ridewood 1903; Giribet and Wheeler 2002).

That the heterorhabdic condition should be independently apomorphic in the Pectinidae and the Ostreidae strongly suggests that, starting with a homorhabdic filibranch condition, there is only one viable basic gill adaptation for particle processing in the high-turbidity habitats in which these two families evolved: heterorhabdy. The considerable selection capacity of this condition (Ward et al. 1998; Cognie et al. 2003; Beninger et al. 2004) indeed constitutes an adaptive advantage for suspensivory in turbid habitats.

Ecological and aquacultural implications

Three periods of major developmental shifts are apparent in *C. gigas* gill ontogeny, summarized in Fig. 8. These are: (1) the change from velum to gill as particle collecting structure, at size 0.35 mm; (2) the change from a V- to a W-shaped gill, coinciding with the acquisition of latero-frontal cirri, at 2.70 mm; and (3) the change from the homorhabdic to the heterorhabdic condition, at 7.50 mm. These developmental shifts may be related to the remarkable increase (nearly 2 orders of magnitude) in grazing rates observed in 1.3 mm and 1.0 cm *Ostrea edulis* (Wilson 1980). Each of these shifts may be expected to be associated with energetic expenditures or functional shifts (Forbes and Lopez 1989), potentially generating periods of increased mortality, depending on the severity of environmental conditions. In the hatchery, where food is abundant and most physico-chemical conditions optimal, increased mortalities at these critical stages may only be observed if other negative factors intrude, such as microbial or parasitic infection. It should be noted that the sizes and ages given here for the critical stages are necessarily derived from hatchery rearings, and are likely to vary in relation to field conditions for natural populations or for oysters cultured from natural spatfall. On the applied level, at the very least, the information of the present study may help to interpret the etiology of larval and juvenile *C. gigas* culture problems.

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