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Demonstration of Nutrient Pathway From the Digestive System to Oocytes in the Gonad Intestinal Loop of the Scallop *Pecten maximus* L.

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Abstract. The mechanism of nutrient transfer from the digestive system to the gonad acini and developing oocytes was investigated in the gonad-intestinal loop system of the queen scallop *Pecten maximus* L. Ferritin was injected directly into the purged intestine of specimens from the wild. Subsequently, a histochemical reaction and transmission electron microscopy were used to localize ferritin in various cell types. Ferritin was rapidly absorbed by the intestinal epithelium, and then appeared in hemocytes in the surrounding connective tissue. In the hemocytes, ferritin was stored in variously sized inclusions, as well as in the general cytoplasm. In all sections examined for the 12 experimental individuals, hemocytes were always found in association with connective tissue fibers extending from the base of the intestinal epithelium to gonad acini. After 30-min incubation, ferritin appeared inside the acini of all individuals. Ferritin-bearing cells were rarely found in association with male acini or gametes, nor with mature female gametes, but often with developing female gametes. Not all individuals showed the same temporal dynamics of ferritin transport, suggesting that nutrient transfer to oocytes is either not a continuous process, or that among individuals, transfer is not synchronized on short time scales. This is the first demonstration of a pathway of nutrient transfer from the intestine, and more generally the digestive system, to developing oocytes in the Bivalvia.

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Introduction

In the Bivalvia, the digestive and reproductive systems are closely situated and often intertwined, either within the visceral mass (the majority of bivalves), or more distinctly separated from the visceral mass, as in the Pectinidae (Galtsoff, 1964; Morales-Alamo and Mann, 1989; Beninger and Le Pennec, 1991; Morse and Zardus, 1997). The ultrastructural characteristics of gametogenesis have only recently begun to be elucidated in this class (Pipe, 1987a, b; Dorange and Le Pennec, 1989; Eckelbarger and Davis, 1996a, b). However, gametogenesis must rely on the transfer of nutrients, which are acquired almost exclusively by other tissues or organs and transferred to the gonad.

Transfer of nutrients from storage or digestive sites to the gonad has been inferred or demonstrated in a number of bivalve species (Goddard and Martin, 1966; Vassallo, 1973; Ansell, 1974; Comely, 1974; Gabbott, 1975, 1983; Adachi, 1979; Zaba, 1981; Lubet *et al.,* 1987; Le Pennec *et al.,* 1991a, b). Although successful gamete production relies on such transfers, very little is known about the underlying pathways and mechanisms. The elaboration of oocyte reserves has been the subject of considerable research in many invertebrates, but is largely lacking in bivalves (see Eckelbarger and Davis, 1996a, for review). Regardless of whether bivalve gametes ultimately elaborate vitelline reserves using autosynthetic (Suzuki *et al.,* 1992) or heterosynthetic pathways (as suggested by Eckelbarger and Davis, 1996a), or both, it is clear that nutrients must be made available, largely from the diet, for the synthesis of the gametes and their reserves.

A summary of known or inferred pathways that transfer nutrients to the gonad acini and gametes has been outlined

for the queen scallop, *Pecten maximus*, based on anatomical, ultrastructural, and histochemical observations (Le Pennec *et al.*, 1991a). In particular, transfer of nutrients from the intestine to the developing gametes was proposed. Although it has long been known that both extracellular and intracellular digestion take place in the intestine of bivalves (Zacks, 1955; Reid, 1966; Payne *et al.*, 1972; Mathers, 1973; Teo and Sabapathy, 1990), the persistence of the "conventional wisdom" that the intestine merely serves as a conduit for undigested matter prompted Purchon (1971) to call for a reexamination of the role of this organ. In the family Pectinidae, the intestine loops within the otherwise anatomically distinct gonad, and indeed Le Pennec *et al.* (1991a) provided data suggesting that nutrients are transferred from this structure to developing gametes. They also proposed a transfer mechanism and pathway involving hemocytes. Enzymatic and detailed ultrastructural studies subsequently showed that the scallop intestinal loop is capable of digestion and assimilation (Le Pennec *et al.*, 1991b). This research provided a framework for the demonstration of transfer pathways using direct physiological techniques such as labeling. In this study, therefore, we have used ferritin as a marker to examine the proposed transfer pathway from the gonad intestinal loop to gonadal tissue in the scallop *Pecten maximus*.

Ferritin is an iron-containing transfer protein, consisting of a core of up to 4300 iron cations in the form of ferric oxyhydroxide and ferric phosphate, and a protein shell of approximately 450,000 Da (Miksys and Saleuddin, 1986). In the specific tissues of living organisms which contain ferritin, the molecules are often grouped into variously sized clusters, with a near-crystalline appearance (Bottke and Sinha, 1979; Miksys and Saleuddin, 1986). A specific stain for iron can therefore be used to distinguish it from other proteins (Bockman and Winbom, 1966; Heneine *et al.*, 1969; Block *et al.*, 1981; Bottke *et al.*, 1982; Boucher-Rodoni and Boucaud-Camou, 1987; Paar *et al.*, 1992; Ito *et al.*, 1992). Ferritin is also visible in uncontrasted transmission electron microscopy (TEM) sections as small, variously sized electron-dense clusters (Bottke and Sinha, 1979; Miksys and Saleuddin, 1986). Ferritin has been used both to demonstrate intestinal absorption mechanisms (Bockman and Winborn, 1966; Boucher-Rodoni and Boucaud-Camou, 1987) and to study mechanisms of uptake into the ferritin-rich yolk of snail oocytes (Bottke *et al.*, 1982). In this study we use ferritin as a substrate model with which to follow the transfer of nutrient molecules from the intestine to the gonadal tissue of *Pecten maximus*. Although hemoglobin is present in the hemocytes of some bivalve families (see reviews by Reid, 1966; Bonaventura and Bonaventura, 1983), none has yet been reported in the pectinids, and in any event, this substance cannot confound histochemical detection of injected ferritin since the iron of hemoglobin cannot be demonstrated histochemically without total destruction of histochemical sections (Kiernan, 1990). Control for the eventual presence of naturally occuring ferritin can be accomplished through the use of control subjects.

Pectinids are ideal candidates for such experiments, because the gonad-intestinal complex is well-separated from the other organs. *Pecten maximus* was chosen in part because it is a simultaneous hermaphrodite, thus allowing investigation of both male and female components within the same individual under identical experimental conditions. The gonad intestinal loop of pectinids also presents the advantage of being easily visible throughout most of the reproductive cycle. No respiratory function has yet been ascribed to bivalve hemocytes, and bivalve plasma generally lacks circulating respiratory pigments (Booth and Mangum. 1978), obviating possible artifacts.

Materials and Methods

Twelve specimens of *Pecten maximus* (size range 9–10 cm shell length, antero-posterior axis) were collected from the Bay of Brest (Finistère, France). The valves of each scallop were kept open with a wedge in the posterior dorsal region, and the proximal part of the descending intestinal loop was located by directing a cold light source at the male portion of the translucent gonad (see Fig. 1). Into this portion of the intestine in each scallop, 1 ml of a 4 mg ml^{-1} solution of cadmium-free ferritin (Sigma horse spleen Type

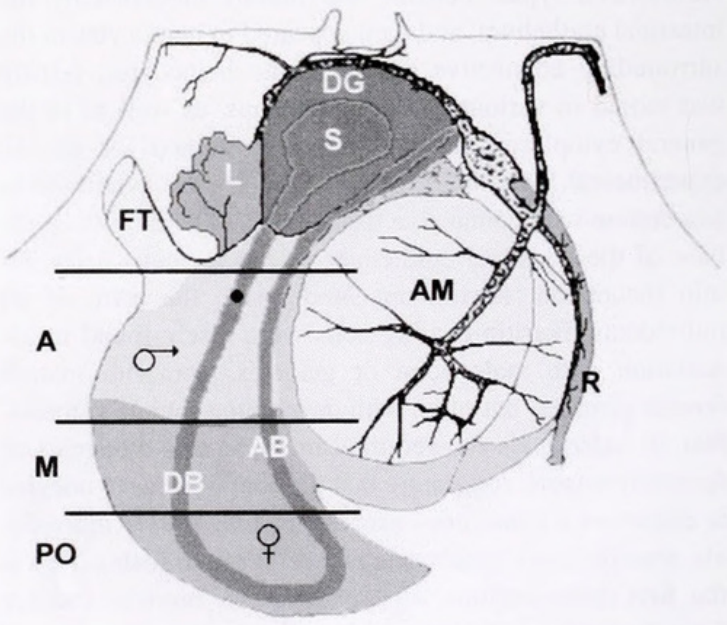


Figure 1. *Pecten maximus*. Schematic diagram to show planes of section in anterior (A), median (M), and posterior (PO) gonad levels. Histological sections were performed on these planes, such that the region of the median level surrounding the descending branch of the intestinal loop (DB) contained predominantly male acini, whereas the region of the median level surrounding the ascending branch of intestinal loop (AB) contained predominantly female acini. AM, adductor muscle; DG, digestive gland; FT, foot; L, lips; R, rectum; S, stomach; ♂, male, and ♀, female parts of gonad.

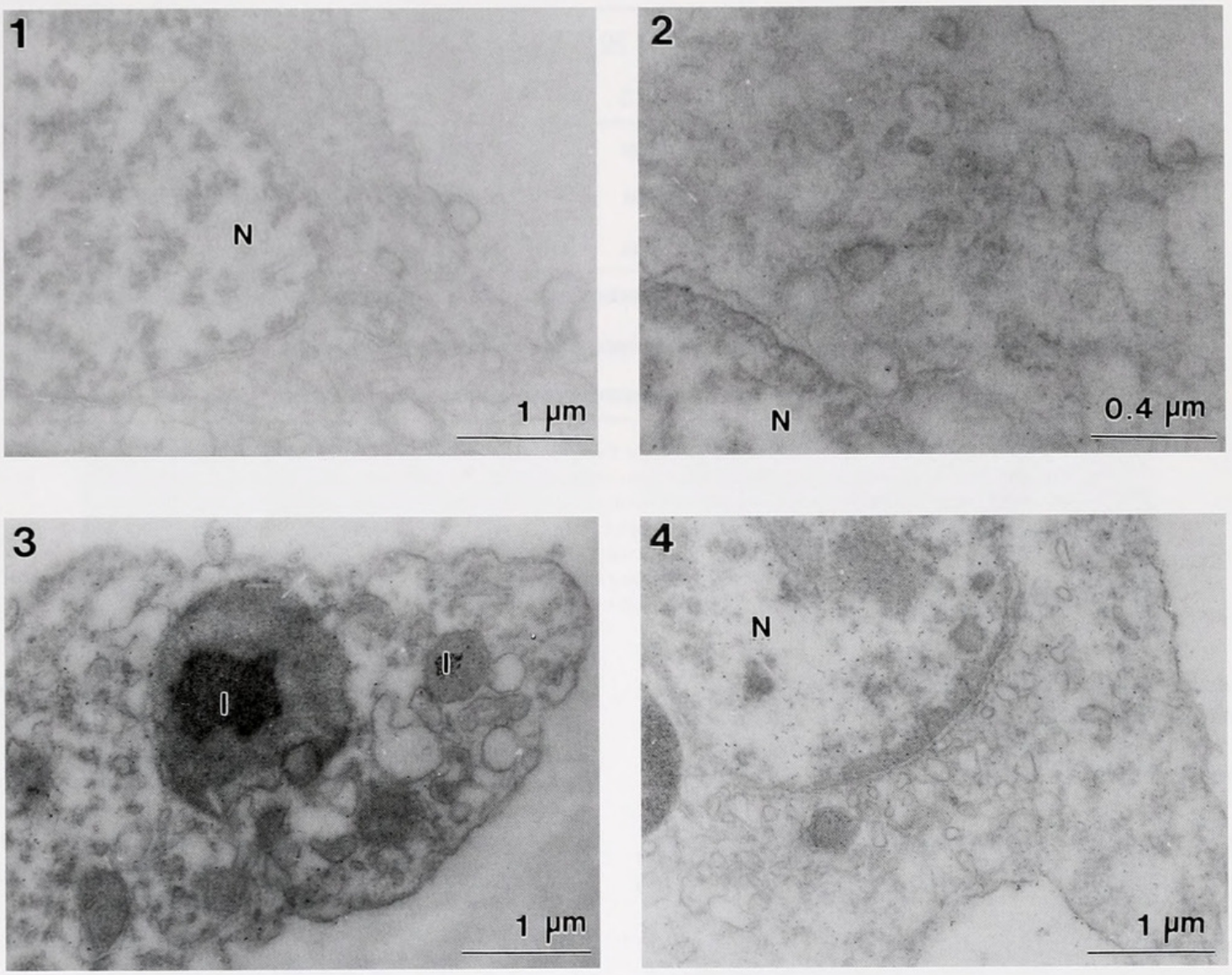


Figure 2. Transmission electron micrographs of uncontrasted oocytes and hemocytes in posterior (female) region of gonad, from individuals which had not been injected with ferritin. **2.1, 2.2** Details of early-developing oocytes at two magnifications (20,000 and 40,000 ×, respectively). **2.3** Detail of rounded hemocyte, showing non-ferritin-containing inclusions (I); **2.4** Detail of pseudopod-bearing hemocyte. Note absence of ferritin clusters in all micrographs. N, nucleus.

1, 10–12 nm diameter molecules) was slowly injected. Complete distribution of ferritin throughout the length of the intestinal lumen was monitored visually by the appearance of the colored solution in the ascending branch. The scallops were divided into four groups of three individuals each, corresponding to 10, 30, 120, and 300 min of exposure to the ferritin solution. Exposure was carried out in 15°C filtered seawater, which was aerated with a pump and airstone. Following the designated period of exposure, the scallops were immediately dissected. Transverse slices of the gonad (about 1–2 mm thick) were removed at three levels along the antero-posterior axis of the gonad as shown in Figure 1, and immediately processed for histology and electron microscopy. To control for the eventual presence of clusters of endogenic ferritin molecules or other electron-dense particles, a control group of three individuals was injected with 0.8 μm filtered seawater, and processed for histology as detailed below.

Histology

Slices of gonad were fixed for histology in aqueous Bouin's solution (24 h), dehydrated in an ascending ethanol-xylene series, and embedded in paraffin. Transverse sections corresponding to each exposure time and gonad level were cut at 5 μm, and each exposure series for a given level were positioned on a single microscope slide. The slides were then immersed in 35% ammonium sulfite for 2 h and rinsed with Milli-Q-filtered ultrapure water (all glassware at this stage was also prerinsed with ultrapure water). Sections were stained using the Turnbull blue protocol,

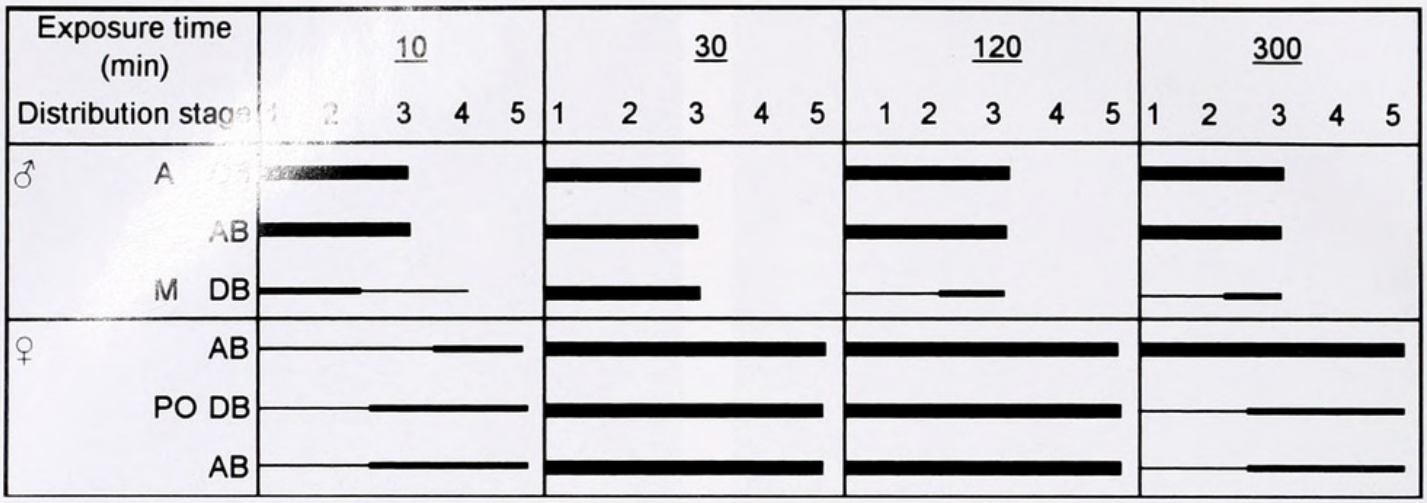


Figure 3. Dynamics of ferritin distribution in the *Pecten maximus* gonad for the three individuals at each exposure time. A, anterior region of gonad; M, median region of gonad; PO, posterior region of gonad; ♂, male portion of gonad; ♀, female portion of gonad; AB, tissues of ascending branch of intestine-gonad complex; DB, tissues of descending branch of intestine-gonad complex; 1, uptake into intestinal epithelium; 2, appearance in hemocytes within connective tissue surrounding intestine; 3, appearance in hemocytes at the outer faces of the acini; 4, appearance in hemocytes within the acini; 5, appearance in hemocytes/follicle cells appressed to oocytes. Thin line represents one individual; medium line represents two individuals; thick line represents three individuals.

counterstained with nuclear red (Gabe, 1968; Vacca, 1985), dehydrated, and mounted under coverslips for photography.

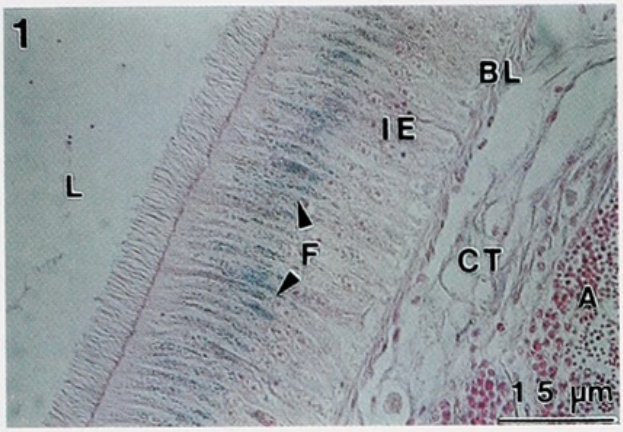
Transmission electron microscopy

For transmission electron microscopy (TEM), the gonad slices were fixed for 10 h in cold (4°C) 2.5% glutaraldehyde −0.1 *M* sodium cacodylate buffer at pH 7.3 and 1100 mOsm. They were then cut into pieces of about 1 mm^3, rinsed twice with the sodium cacodylate buffer solution, and post-fixed in cold 1% osmium tetroxide in buffer for 1 h. The tissue pieces were then rinsed with 70% ethanol, dehydrated in an ascending ethanol-xylene series, and embedded in Epon 812 resin. Transverse sections (400–600 nm) were cut with a Reichert ultracut-S ultramicrotome and examined without further contrast using a JEOL 100CX transmission electron microscope. The lack of further contrasting allowed ferritin granules to be identified without ambiguity in the resulting micrographs. However, lack of contrast and the very small size of the free ferritin molecules and masses render observation *via* the fluorescent TEM screen somewhat challenging.

Results

As will be shown below, it is important to distinguish between oocytes in different states. Oocytes may be in development (previtellogenic and vitellogenic oocytes), or

Figure 4. *Pecten maximus*. Histological sections showing the presence of ferritin in the intestine and gonad. Turnbull blue-neutral red staining protocol. **4.1** Low-magnification view, after 120-min incubation, of ciliated intestinal epithelium (IE) in the anterior (male) gonad level with assimilated ferritin (F), surrounding connective tissue (CT), and adjacent acini (A) in the male region of the gonad. BL, basal lamina; L, lumen of intestine. **4.2** Basal region of intestine, in median (male + female) level of gonad after 10-min incubation, showing ferritin-containing hemocytes (HF) associated with connective tissue (CT) surrounding basal lamina (BL) of intestine. **4.3** Ferritin-containing hemocytes (HF) associated with connective tissue fibers (CF) leading from the basal lamina of the intestine to gonad acinus (A) in median (male + female) level of gonad. Incubation time: 120 min. **4.4** Ferritin-containing hemocytes (HF) positioned both at the base (→) and on the inside of gonad acini (▶), in posterior (female) level of gonad. Note proximity and association of hemocytes with developing (DO) and vitellogenic oocytes (VO) within the acinus (A). Incubation time: 30 min. **4.5** Ferritin-containing hemocytes (HF) within acinus (A) of posterior (female) level of gonad. DO, developing oocyte; RMO, residual mature oocyte. Incubation time: 30 min. **4.6** Ferritin-containing hemocytes (HF) attached to developing oocyte (DO) within acinus (A) of posterior (female) level of gonad. RMO, residual mature oocyte. Incubation time: 30 min. **4.7** Multiple large, round, ferritin-containing hemocytes (HF) attached to developing oocytes (DO) within acinus (A) in posterior (female) level of gonad. RMO, residual mature oocyte. Incubation time: 30 min. **4.8** Residual vitellogenic oocytes (RVO, late-developing stage) within an acinus (A) in posterior (female) level of gonad. Note attached large, round, ferritin-containing hemocytes/follicle cells (HF). CT, inter-acinal connective tissue. Incubation time: 120 min.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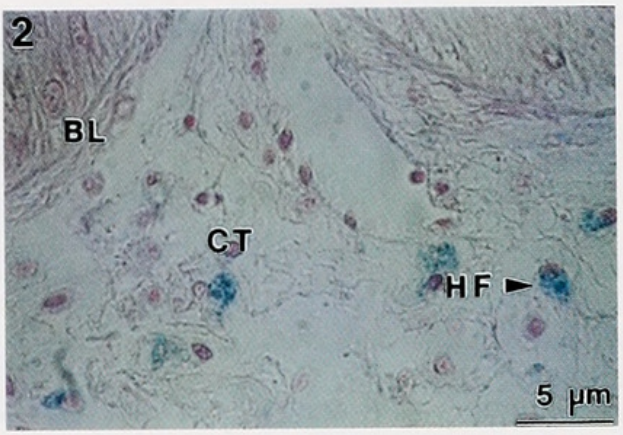
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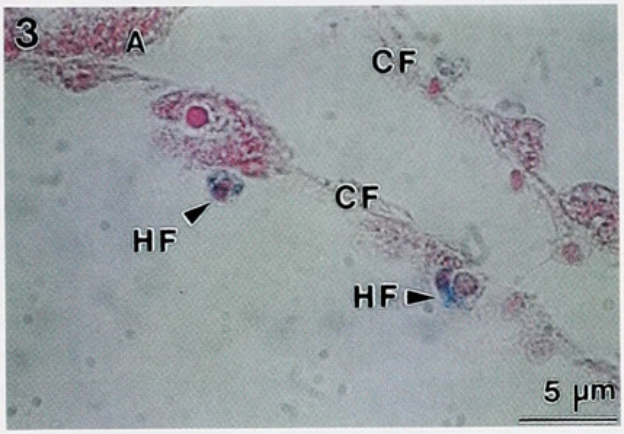
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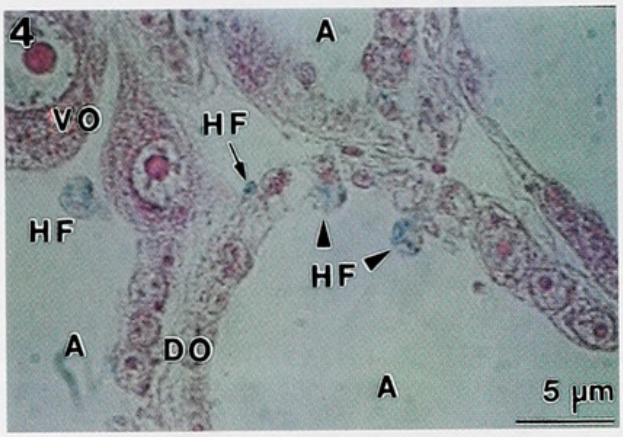
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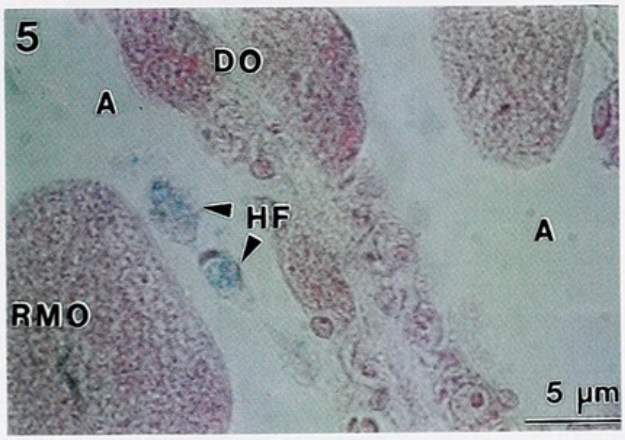
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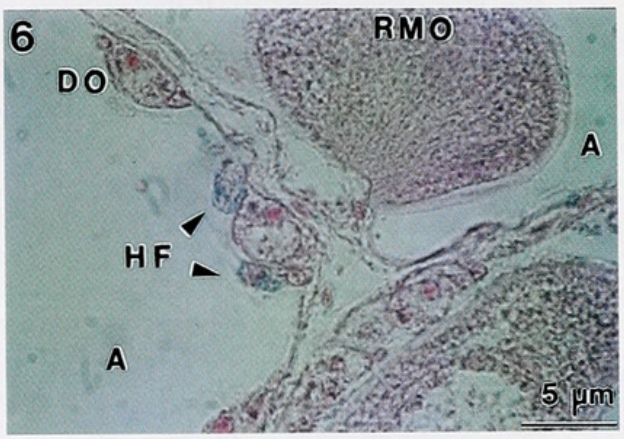
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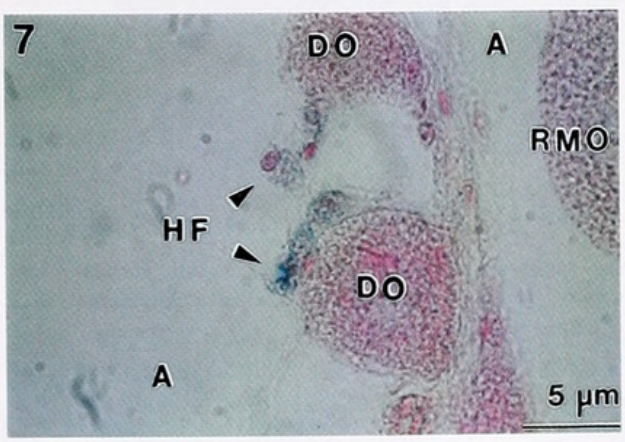
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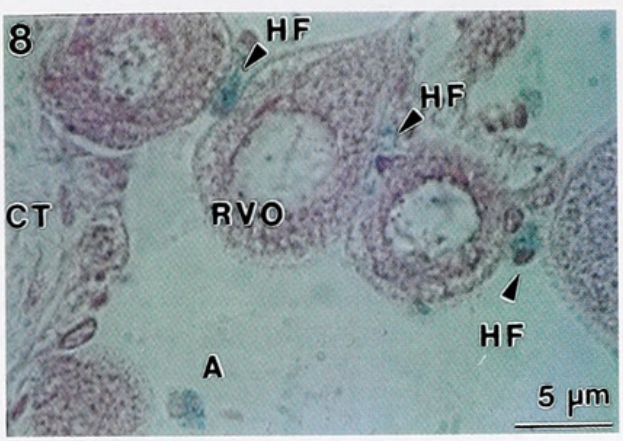
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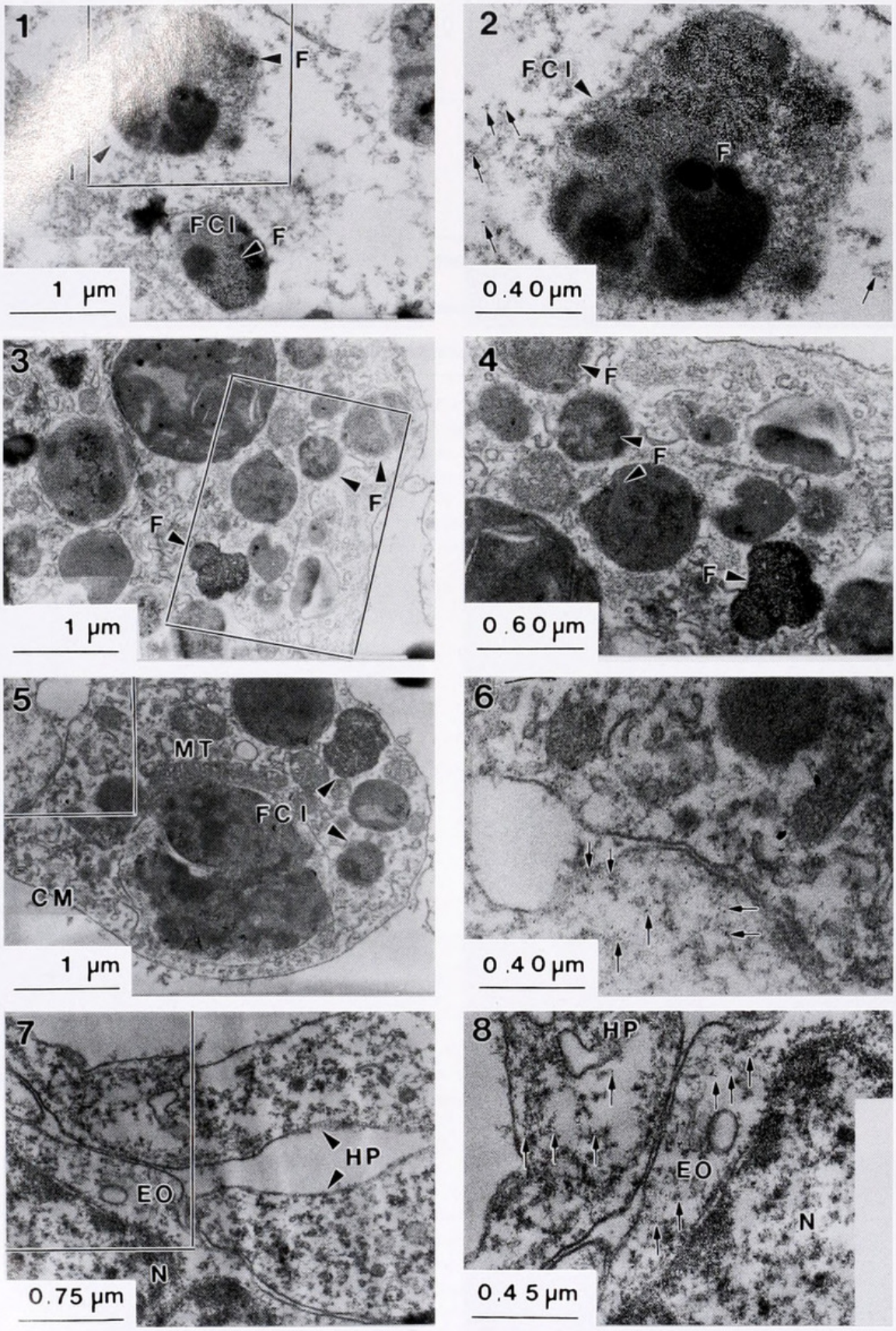

Figure 5. *Pecten maximus.* Uncontrasted transmission electron micrographs of cells in the intestine-gonad complex following ferritin injection in the intestinal lumen. **5.1** Detail of cytoplasm of absorptive cell from intestinal epithelium, in median (male + female) level of gonad showing ferriting clusters (F) in inclusions (FCI). 5.2 Enlargement of indicated region in Fig. 4.1. Note presence of ferritin both within the inclusions and distributed freely in the cytoplasm (→). **5.3** Detail of a large, rounded hemocyte in the connective tissue surrounding the acini in median (male + female) level of gonad. Note presence of ferritin (F) in variously sized inclusions. **5.4** Enlargement of region indicated in Fig. 4.3., showing ferritin (F) in inclusions. **5.5, 5.6** Portion

they may be mature (detached from the acinal wall). Following spawning, some oocytes of both types may remain in the acinus; these are termed residual oocytes. We will thus adopt this terminology in the present paper.

No ferritin was detected using either the Turnbull method or TEM observation (Fig. 2) in any of the control individuals; we may thus conclude that the ferritin observed in our histological and electron microscopical sections was injected.

The histological observations of the entire set of individuals and gonad levels revealed that the distribution of ferritin in the sampled tissues could be divided into 5 sequential steps: (1) uptake into intestinal epithelium, (2) appearance in hemocytes among the connective tissue surrounding the intestine, (3) appearance in hemocytes at the exterior faces of acini, (4) appearance in hemocytes within the acini, and (5) appearance in hemocytes/follicle cells appressed to oocytes. It is not possible to ascertain whether these cells were hemocytes or follicle cells in the histological sections (only TEM profiles can distinguish these cell types). The TEM profiles of these cells described below do not correspond to follicle cells, which are rich in rough endoplasmic reticulum (Dorange and Le Pennec, 1989), absent in the two pectinid hemocyte types (Beninger and Le Pennec, 1991) and in the cells observed appressed to the oocytes; however, as the TEM sections were uncontrasted, it is not possible to distinguish these cells with certainty. Although ferritin-containing hemocytes were very rarely observed associated with male spermatogonia, they were never observed appressed to developing male gametes. The distribution of ferritin among the sections is summarized in Figure 3, which presents each step in the sequence, for each gonad level, and each intestinal branch (ascending and descending), for each exposure time.

Light microscopy of the histological sections showed that ferritin was distributed in cells of the intestinal epithelium at all three antero-posterior levels of the gonad-intestinal complex, from the apex to the basal region of the columnar intestinal cells (Fig. 4). Ferritin appeared very rapidly in the intestinal cells, and was observed in all preparations, even after 10 min of exposure. TEM micrographs revealed that ferritin appeared predominantly in variously sized inclusions of the intestinal cells, although isolated granules could also be found in the cytoplasm (Fig. 5.1, 5.2). Large, round, ferritin-containing hemocytes were also detected beneath the intestinal basal lamina within the surrounding connective tissue after only a 10-min exposure (Fig. 4.2). Ferritin appeared in these hemocytes in variously sized inclusions, as well as being generally distributed within the cytoplasm (Fig. 5.3–5.6). In all sections examined for the 12 experimental individuals, ferritin-containing hemocytes were always observed in close association with connective tissue fibers extending from the basal lamina of the intestinal epithelium and the acini (Fig. 4.2, 4.3).

Further transport of ferritin to the gonad acini and oocytes appeared to be somewhat independent of exposure time (Fig. 3). Specimens in which further ferritin transport was observed showed ferritin-containing hemocytes/follicle cells both at the outside faces of gonad acini (including those which were remote from the intestine) and inside the gonad acini (Fig. 4.3–4.8). These hemocytes/follicle cells were typically found appressed to developing oocytes (Fig. 4.3–4.8). Although no positive Turnbull blue reaction was observed in female gametes, transmission electron micrographs revealed dispersed ferritin clusters within the cytoplasm of oocytes to which ferritin-containing hemocytes/follicle cells were appressed (Fig. 5.7, 5.8).

The localization and distribution of ferritin within the various cell types involved in the transfer sequence presented notable differences, which also explains the absence of a visibly positive Turnbull reaction in some cell types. Both the intestinal cells and the transport hemocytes possessed variously sized inclusions with considerable concentrations of ferritin, and these cells presented visibly positive Turnbull reactions.

Upon histological examination, 3 of the 12 experimental scallops were observed to be mature and ready to spawn, while the remaining 9 had already spawned and had begun producing a new cohort of female gametes. Although ferritin appeared in the intestinal epithelium and surrounding connective tissue of the mature scallops, ferritin-containing hemocytes were virtually never observed, either in the acini, or appressed to the oocytes of these individuals. Moreover, despite the presence of mature residual oocytes in the acini of the individuals which had spawned previously, ferritin-containing hemocytes/follicle cells were never observed appressed to them. Ferritin-containing hemocytes/follicle cells were, however, observed appressed to late-developing residual oocytes in these individuals (Fig. 4.8).

Discussion

The uptake of ferritin in the intestinal epithelium of *Pecten maximus*, observed in the present study, demon-

of a large, rounded hemocyte within an acinus in posterior (female) level of gonad, showing ferritin-containing inclusions (FCI), as well as ferritin molecules and clusters distributed freely in the cytoplasm (→). CM, cell membrane; MT, mitochondria. **5.7, 5.8** Detail of association between early developing oocyte (EO) and pseudopods of hemocyte/follicle cell (HP), in posterior (female) level of gonad. Note ferritin freely distributed in cytoplasm of both cells (→). N, nucleus.

strates that proteinaceous substrates are absorbed by the scallop intestinal epithelium, at least some of which subsequently appear in hemocytes at the cell bases. The rapidity and ubiquity of this uptake, observed in the present study, as well as the cytological and enzymatic equipment of the intestinal epithelium (Le Pennec *et al.,* 1991b), suggest that the scallop intestine is well-adapted for both a digestive and a transfer function. This result is consistent with the view of the intestine as a digestive organ in bivalves (Zacks, 1955; Reid, 1966; Payne *et al.,* 1972; Mathers, 1973; Teo and Sabapathy, 1990). The development of the gonad around the intestine optimizes the potential for the transfer of nutrients to developing gametes.

The results of the present study allow us to identify the various cell categories and pathways that mediate the entero-gonadal transfer system in bivalves: intestine epithelial cells; large hemocytes which concentrate ferritin in cytoplasmic inclusions, in addition to that present freely in the cytoplasm; and connective fibers which are often associated with the hemocytes. Previous studies have shown that hemocytes may move across the intestinal epithelium: those containing material of little or no nutritional value move toward the lumen, while those containing nutritionally valuable material move from the lumen to the tissues surrounding the intestinal epithelium (see Cheng, 1996, for review of bivalve hemocyte types and functions). The present study shows that the scallop intestinal cells may, themselves, move nutrients from the lumen to the basal lamina; hemocytes subsequently act as transport vectors to the surrounding gonad tissue. This line of investigation does not seem to have been pursued previously, despite long-held anatomical knowledge of the scallop intestine-gonad relationship.

The pathway of intestine-oocyte transfer seems to conform largely to that postulated by Le Pennec *et al.,* based on detailed histological observations (1991a). These authors proposed that nutrients assimilated by the intestinal epithelium are transferred to hemocytes at the base of the basal lamina, as observed in the present study. They further proposed that the efficiency of this transport relied upon connective tissue fibers linking the basal lamina to the acini, such that incorporated nutrients could be directed specifically to acini. In the present study, ferritin-containing hemocytes were always observed in association with connective tissue fibers between the base of the intestinal epithelium and the bases of the acini.

The asymmetry in ferritin distribution between the male and female parts of the simultaneous hermaphroditic gonad is consistent with the difference in the composition and consequent energetic demand of male and female gametes. While ferritin-containing hemocytes/follicle cells were readily observed appressed to developing oocytes, which elaborate substantial vitelline reserves, they were rarely observed within male gonad acini, which produce small spermatozoa with few energy reserves (see Beninger and Le Pennec, 1991, for the sizes of spermatozoa and oocytes in pectinids, and Beninger and Le Pennec, 1997, for the sizes of spermatozoa and oocytes in bivalves generally); and ferritin-containing hemocytes were never observed appressed to developing male gametes. This asymmetry suggests that the entero-gonadal pathway is specific to female gametes. Another such possible distinction is described below.

The fact that ferritin-containing hemocytes/follicle cells were always found in association with developing oocytes, and never in association with mature oocytes, suggests that the ferritin-containing cells might be able to distinguish between these two states, and can supply nutrients to the oocytes most in need, *i.e.,* developing oocytes. It should be noted that the follicle cells detach from the mature oocytes of *Pecten maximus* (Dorange and Le Pennec, 1989). Although both residual mature and residual developing oocytes were observed in individuals that had recently spawned, ferritin-containing cells were only observed appressed to the residual developing oocytes, and never to the residual mature oocytes. This finding suggests that the spawning status (*i.e.,* prespawning or postspawning) does not influence nutrient transport; rather, the oocyte developmental stage appears to be the determining feature of such transport, even when these gametes are destined for atresia and metabolic recycling (Pipe, 1987b; Dorange and Le Pennec, 1989; Le Pennec *et al.,* 1991a). The data of Figure 3 show that ferritin transfer to developing oocytes does not occur at a uniform rate for all individuals; indeed, after 300 min, some individuals had no ferritin-containing cells appressed to developing oocytes. While this could be due to the stress induced by the experimental procedure, it could also indicate that nutrient transfer from the intestine is not a continuous physiological activity, or that among individuals, transfer is not synchronous on such short time scales. We are unaware of any studies that present the dynamics of oogenesis on such short time scales, but this is an interesting physiological question.

While the particular scallop gonad-intestine anatomical relationship is not common in bivalves, the digestive system and gonad are generally closely associated and intertwined, with loose connective tissue containing abundant fibers between these epithelia (Galtsoff, 1964; Morales-Alarno and Mann, 1989; Morse and Zardus, 1997). These similarities to the pectinid system suggest that transfer from digestive epithelia to developing oocytes *via* a pathway similar to that described above may be a general feature of bivalve physiology.

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previous unpublished experiments on this subject, which guided the methodology of the present work. We are indebted to the staff of *The Biological Bulletin* for their very rigorous editorial work on this paper, which allowed us to substantially improve earlier versions of the manuscript. As a study of no direct economic or medical impact on humans, it is not possible to acknowledge funding from any of the French funding agencies.

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