Chapter 7

The Circulatory System

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INTRODUCTION

The eastern oyster, in common with other species of bivalves, possesses an open-type circulatory system. The systemic heart pumps hemolymph into arteries that branch and ultimately open into sinuses; the latter are contiguous intercellular spaces filled with fluid and collectively represent the hemocoel. Hemolymph evolved from the intercellular fluid of the mesenchyme of Platyhelminthes that was moved randomly by contraction of body muscles. Subsequently, some intercellular spaces of the mesenchyme enlarged and became contiguous to form sinuses; these spaces represent a persistent embryonic blastocoel. In the Rhynchocoela, muscular vessels evolved that connected with the sinuses and served to move the hemolymph under some pressure. In the Mollusca, a portion of the dorsal vessel became specialized for contraction and evolved into the systemic heart. The hemocoel in the mollusca enlarged at the expense of the coelom which then became restricted to three areas: the gonocoel, the renocoel, and the pericardial cavity. This process by which the hemocoel encroached on the coelom was first elucidated by Lankester (1888, 1909) who termed it phlebedesis.

The vascular system of oysters has been investigated for more than 300 years starting with the work of Willis (1672) on the heart of the European flat oyster, *Ostrea edulis*. More than 200 years elapsed before Brooks (1880) described the beating heart in the eastern oyster. Kellogg (1892) mentioned the shape of the heart and discussed the striated nature of cardiac muscle fibers. Further, he explained that the atria had their distinctive brown color due to the presence of pigmented cells. He also correctly described the positions of the anterior and posterior aortae. He discoursed upon hemolymph vessels of the gill principal filaments as well as their connections to the plical hemolymph sinus.

Moore (1897) discussed and accurately figured the heart and pericardium as well as the position of two of the three veins that return hemolymph to the heart. Federighi (1929) plotted heart rate in intact oysters at various temperatures. Stauber (1940) investigated the rate of heart beat in oysters with open and closed valves and reported greatly slowed heart rate in closed oysters. Eble (1958) presented the circulation of hemolymph in the gill of the eastern oyster and Eble (1963) described in detail the complete circulatory system of this species. Galtsoff (1964) described aspects of the circulatory system and discussed hemocyte cytology and physiology.

The circulatory systems, including hearts, of other oyster species from around the world, have been studied extensively (*Ostrea edulis*, Bonnet 1877; Menegaux 1889, 1890; Janssens 1893; Ridewood 1903; Elsey 1935; Hopkins 1934a, b; 1936a, b; the Portuguese oyster, *Crassostrea angulata*, Leenhardt 1926).

HEMOLYMPH VESSELS — NOMENCLATURE AND GENERAL STRUCTURE

I have named vessels supplying or draining a particular organ after that organ or area, although whenever possible I have used the same vessel names as those already used for other species of oysters (Mene-

gaux 1889; Leenhardt 1926). Also, because most vessels branch repeatedly to supply organs in the visceral mass, I have called them "visceral" vessels and named them according to their position (e.g., anterior, posterior, ventral) in the visceral mass. Abbreviations used in the figures are explained in the Appendix. Unless otherwise indicated, means are presented ± 1 SD.

Arteries conduct hemolymph away from the systemic heart; they have well-formed walls that are eosinophilic (Fig.1A, B) and on which scattered nuclei may be seen adhering to the luminal surface; whether these are nuclei of endothelial cells or of hemocytes is not clear but nuclear cytology suggests they are not from hemocytes. The surface of the arterial wall that abuts the Leydig tissue is not smooth but extends between Leydig cells as fine strands. Veins conduct hemolymph back to the systemic heart; they have a much thinner lining (Fig. 2). Sinuses, as described, are intercellular spaces (Fig. 1A, B); hemolymph directly bathes the tissue cells. The transition from an artery to a sinus is gradual (Fig. 1A, B). A special type of sinus, that which surrounds the alimentary canal, is characterized by many slender cells that extend from the basement membrane of the gut across the sinus to connect with adjacent Leydig tissue (Fig. 1A, B).

In addition to the systemic heart, the eastern oyster has paired accessory hearts that pump hemo-

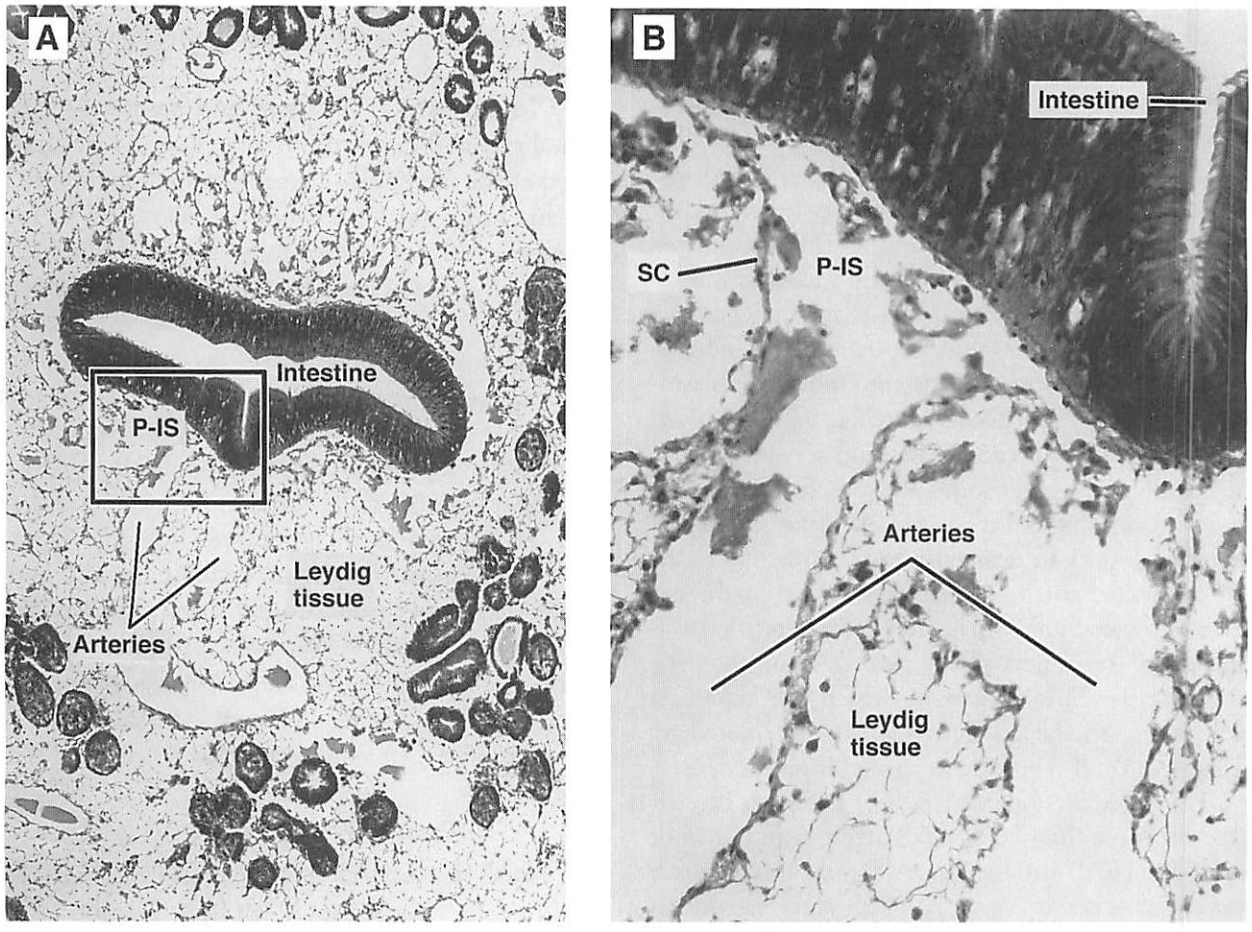


Figure 1. Arterial supply to the intestine. (A) Note two small arteries supplying hemolymph to the intestine; they terminate in a large sinus, the peri-intestinal sinus [P-IS], that bathes the intestine. Horizontal field width (HFW) = 967 µm. (B) Enlargement of area in rectangle in (A). [SC] slender cell. HFW = 305 µm.

lymph from renal sinuses into the mantle; however, it has no respiratory pigment either dissolved in the hemolymph or contained in hemocytes.

PERICARDIUM, HEART, AND AORTAE

Pericardial Coelom

The pericardial coelom is a thin-walled chamber that lies between the visceral mass and the adductor muscle. It is a specialized multifunctional region of the coelom that protects the systemic heart and provides room for its muscular activity while housing and protecting the anterior and posterior aortae. As a fluid-filled cavity, the pericardial coelom serves to dampen the sudden surge of hemolymph that fills the ventricle when the adductor muscle contracts. It collects ultrafiltrate from the atria and passes this material to the kidney via the reno-pericardial canal. The asymmetrical pericardial coelom extends further along the right anterior surface of the adductor muscle than the left. The volume of pericardial fluid in oysters 12 to 14 cm in shell height varies from 2.4 to 2.7 ml (Galtsoff 1964).

The pericardial coelom is delimited by the pericardium which consists chiefly of collagenous connective tissue, hemolymph sinuses, and Leydig cells with epithelia on both surfaces. Based on my examination of histological material, I have found that the internal epithelium is simple, low cuboidal tissue interspersed with granular cells of the pericardial gland (Fig. 3). Externally, on the left side where the pericardium fuses with the mantle, the epithelium is contiguous with and identical to the shell-side pallial epithelium but on the right side where the pericardium forms the internal wall of the promyal chamber, the external epithelium is a simple, nonciliated high cuboidal type containing patches of ciliated, glandular cells rich in neutral glycoproteins (Fig. 3).

Systemic Heart

The systemic heart consists of three chambers, two atria and a common ventricle, suspended obliquely in the pericardial coelom. The paired atria contain cells of the pericardial glands and are colored light to dark brown. The atria receive hemolymph from three veins and, in turn, empty into the ventricle; the latter runs in a dorsoanterior direction in the pericardial coelom and gives rise to the anterior and posterior aortae (Fig. 4A). The ventricle is separated from the atria by a prominent constriction, the atrioventricular (A-V) junction, and is visually demarcated from the atria by its coloration: it is white in

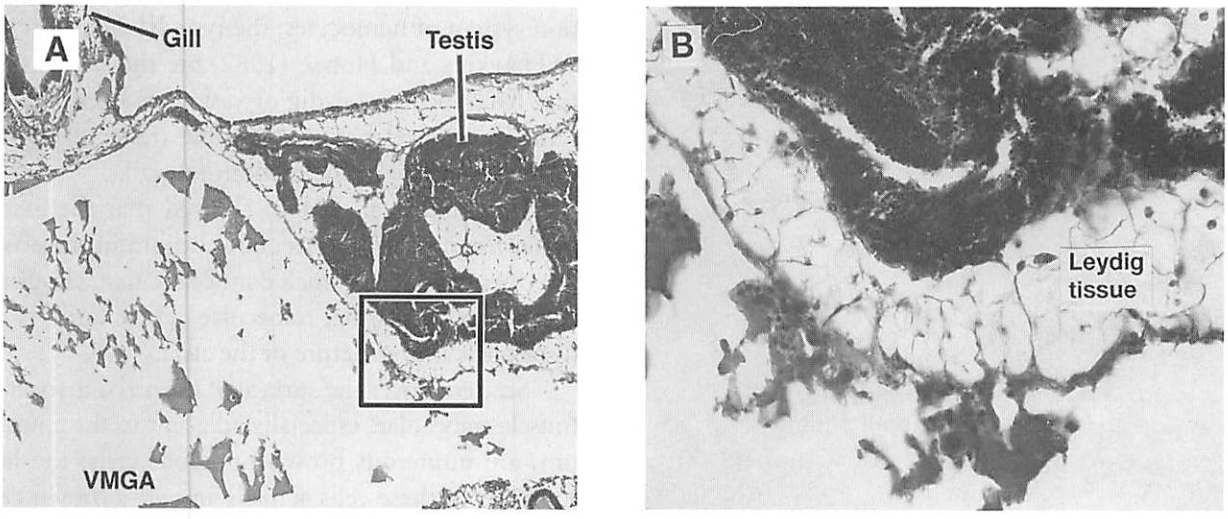


Figure 2. (A) Histology of the medial gill axis vein [VMGA]. Large veins such as this have a lining similar to arteries but much thinner. HFW = 940 µm. (B) Enlargement of area in rectangle in (A). HFW = 224 µm.

stark contrast to the brown atria. The ventricle is pear shaped and bears a slight constriction down the middle of its length. Two pairs of A-V valves, one pair associated with each atrium, are located at the A-V junction; these valves are two muscular flaps that originate in the tissues of the atrio-ventricular junction and project into the lumen of the ventricle (Fig. 5). They are forced open by hemolymph coming from the atria but are pressed together by hemolymph under pressure when the ventricle contracts thereby preventing the regurgitation of hemolymph back into the atria.

The epicardium of the atria is a simple, low columnar epithelium that rests on a distinct basement membrane (Fig. 6). Seen at low magnification with the scanning electron microscope, the epicardium is organized in rows and furrows that run perpendicular to the long axis of the heart. Epicardial cells are joined laterally by desmosomes and by septate desmosomes. The pericardial surface of epicardial cells is covered with microvilli (Fig. 6); Ruthenium red staining reveals a poorly developed, punctate cell coat (Hawkins et al. 1980). Podocytes (large cells with foot-like processes) are found between epicardial cells and the basement membrane; they have large areas of fenestration, broad peripodocyte cisternae (large intracellular spaces between the foot-like processes) and few granules. Podocytes were never observed to be directly apposed to the pericardial fluid and no direct channels from the peripodocyte space to the pericardial cavity have been observed (Hawkins et al. 1980).

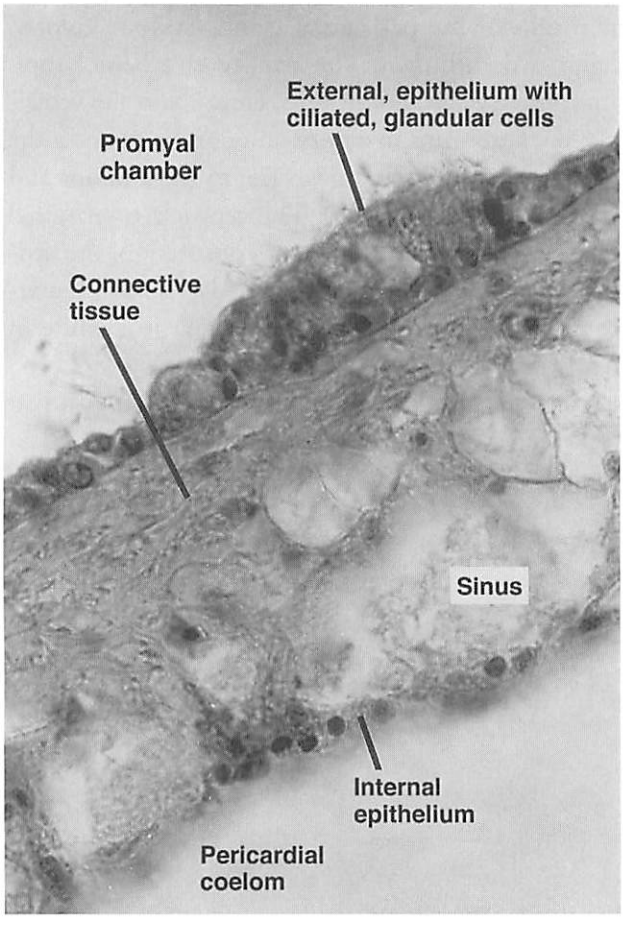


Figure 3. Histological section of right pericardium. HFW = 41 μm.

The atrial myocardium consists of a trabeculated network of "striated" muscular fascicles. Attached to muscle trabeculae in large areas are broad, flattened perforated cells that give rise to numerous thin processes and bridges (Hawkins et al. 1980). An associated layer of connective tissue consists of many fine, interwoven fibrils. The broad, flattened cells were identified by Hawkins et al. (1980) as type II agranular hemocytes originally described by Feng et al (1971); Hawkins and Howse (1982) also describe a type III agranular hemocyte that is closely applied to myocardial trabeculae and was the only cell in their study to contain phagocytosed cellular debris. Cheng in Chapter 8 maintains that in his classification system of hemocytes, the type III agranular cells of Hawkins and Howse (1982) are the same as mature hyalinocytes. Leydig or vesiculated cells are present, interposed between muscle trabeculae where the latter anchor in the myocardial wall.

Hawkins et al. (1980) showed that the eastern oyster has no continuous endocardium in the atrium but does have a very thick connective tissue layer that covers the myocardial trabeculae. These authors also discuss the fine structure of the atria.

Scattered over the surface of the atrial myocardial muscle trabeculae, especially adjacent to the epicardium, are numerous brown or serous cells; the large numbers of these cells actually impart a brown color to the atria. Brown cells are spherical to ovoid-free cells, 12 to 20 μm in diameter with an eccentric nucleus, 3 to 5 μm in diameter; they are characterized

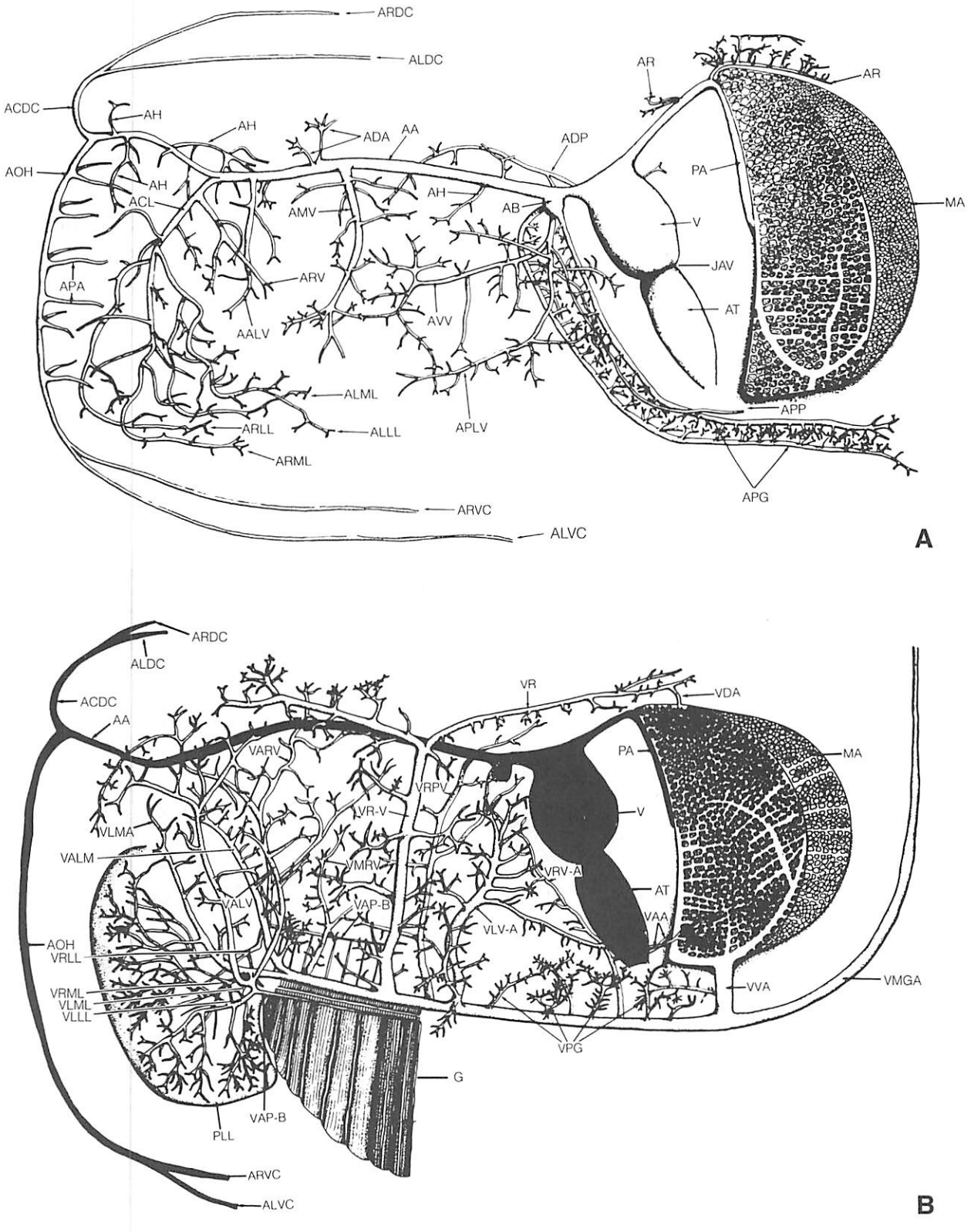


Figure 4. Composite drawings (× 2.4) of (A) the arterial system viewed from left side and of (B) the venous system viewed from the left side. See appendix (p. 298) for list of abbreviated terms.

by the presence of large cytoplasmic granules, fenestration of the outer or plasma membrane, and an extensive tubular network originating in, or emptying into, the plasma membrane fenestration (Ruddell and Wellings 1971). Rifkin et al. (1969) noted that brown cells typically have a leached appearance when viewed with the transmission electron microscope; this has been confirmed by Ruddell and Wellings (1971). Large arrays of rough endoplasmic reticulum are lacking. Glycogen deposits are always in the form of beta particles, never the alpha variety[1] (Ruddell and Wellings 1971).

Brown-cell ultrastructure in the Pacific oyster *Crassostrea gigas* is a function of season of the year. During the summer, oysters have a well developed and extensive Golgi apparatus that is rarely observed in winter and brown cells collected in winter (water temperature less than 12°C) have long, tubular mitochondria not seen during the summer (Ruddell and Wellings 1971). Details of the fenestrations of brown-cell fine structure are discussed by Ruddell and Wellings (1971). Cheng (see Chapter 8) discusses the possible origin and functions of these interesting cells especially as they relate to several oyster diseases.

The histology of the ventricle is very similar to that of the atrium. The epicardium consists of simple

[1] The term beta glycogen deposits refers to individual glycogen particles, whereas in alpha deposits the glycogen particles are juxtaposed in a rosette formation.

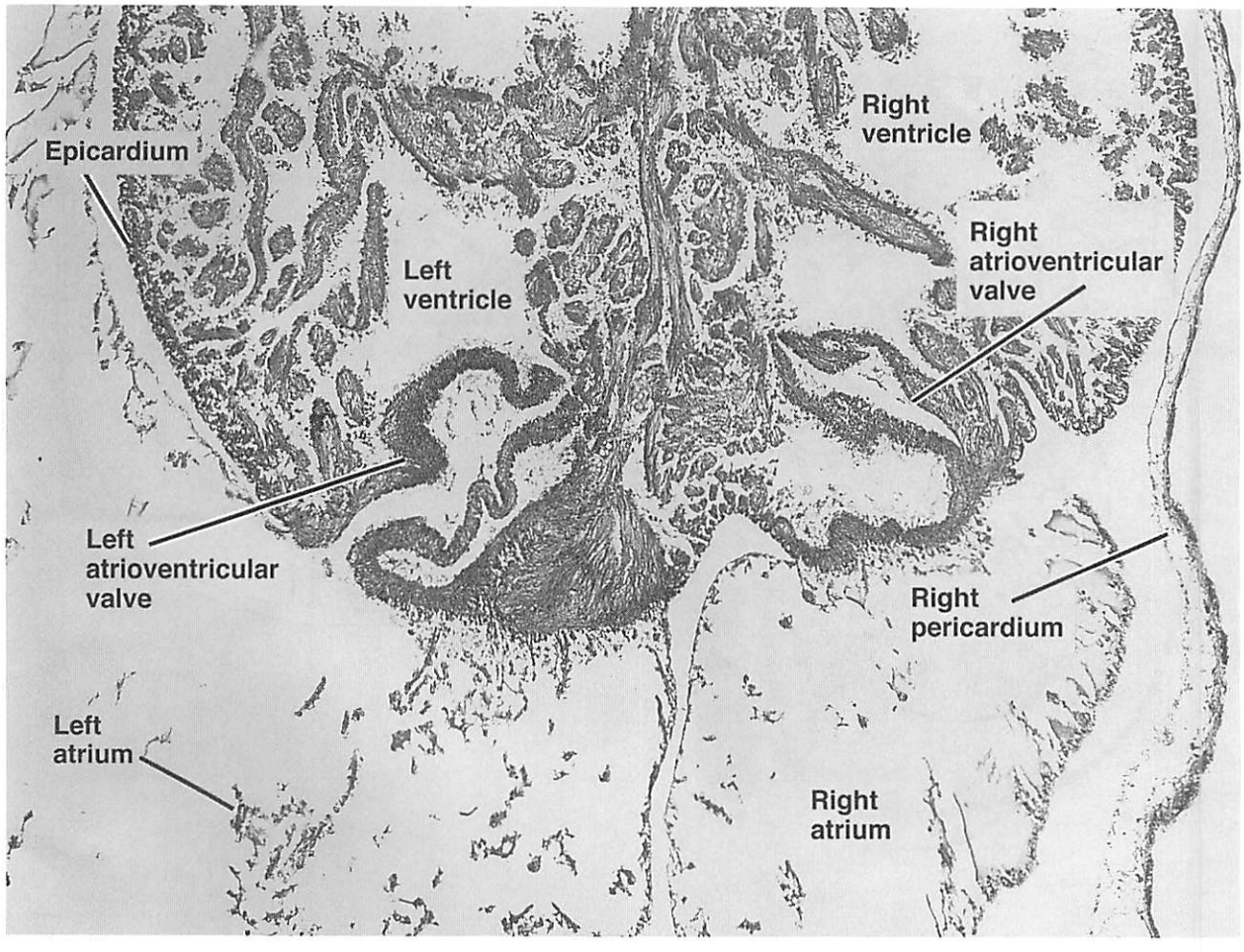


Figure 5. Histological section through the systemic heart to show left and right atrioventricular valves. HFW = 1.5 mm.

cuboidal cells that rest on a basement membrane (Figs. 5, 7). The myocardium is exceptionally well developed and consists of stout muscle trabeculae that form a dense network of fibers especially near the epicardium (Figs. 7, 8). Interposed among myocardial trabeculae in this region are many Leydig cells that, like myocardial fibers, store glycogen.

Myocardial fibers have a thin cell coat that compares in thickness to that found in vertebrates (Hawkins et al. 1980). It is difficult to ascertain whether a continuous endocardium is present in the ventricle of the eastern oyster because so many hemocytes adhere to the myocardial fibers that their surface is obscured (Fig. 8). More research is needed to elucidate the structure and function of both the vesicular-type connective tissue and the endocardial layer.

Physiology

Heart rate is a direct function of ambient temperature but other factors such as valve closure, oxygen tension, and salinity can modify the frequency of beat. Federighi (1929) reported rates as high as 47 beats min^{-1} at 25°C. Lower rates (20 beats min^{-1} at 20°C) were reported by Koehring (1937). Lowe (1974) measured heart rate and temperature in the mantle cavity of intact *Crassostrea gigas* and the soft-shell clam, *Mya arenaria*. He concluded that heart rate was controlled by thermoreceptors located in the mantle cavity, possibly in pallial tentacles, because heart rate closely followed temperature changes in the mantle cavity. Walne (1972) found heart rate to be a direct function of the oyster's (*Ostrea edulis*) pumping rate. An oyster of 0.94 g dry weight showed an in-

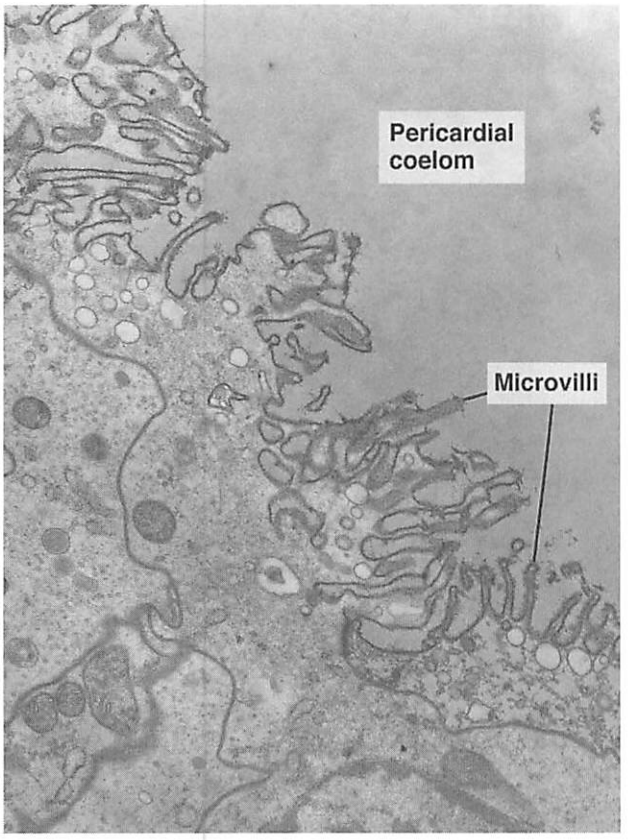


Figure 6. Transmission electron micrograph of atrial epicardium showing thin cell coat of microvilli stained with Ruthenium red. × 29,890. From Hawkins et al. (1980).

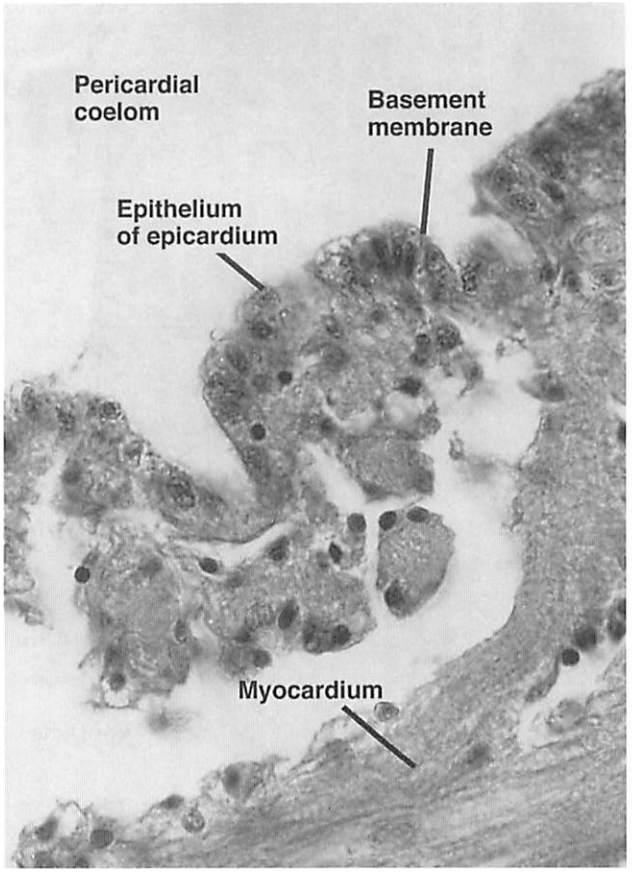


Figure 7. Histological section through ventricular epicardium and associated myocardium. HFW = 78 μm.

crease of 17% heart rate for a 300% increase in water filtration rate. Basically, heart rates from 17 to 20 beats min^{-1} were directly proportional to pumping rates from 100 to 400 ml min^{-1}. Brand (1968) and Taylor (1974) canulated the mantle cavities of the freshwater unionid, *Anodonta anatina*, and the ocean quahog, *Arctica islandica,* and perfused water of various oxygen tensions through the mantle cavity of fully adducted animals; they found that heart beats always responded to the oxygen tension of the fluid in the mantle cavity rather than to any mechanical effect of shell closure (Bayne et al. 1976).

Stauber (1940) reported heart rates of 14 to 16 beats min^{-1} at 17.5°C with the valves open; when the valves closed the heart rate became irregular and slowed to 3 to 6 min^{-1}, sometimes stopping for a period of 2 to 3 min immediately after shell closure. This is similar to reports for other bivalves (see Coleman [1971] for a review).

Galtsoff (1964) reported that heart tissue of *C. virginica* stained with methylene blue showed many neuron cell bodies and fibers; further, he stated that the distribution of these cells was similar to that in the Kokegoromo oyster, *Striostrea circumpicta, C. gigas,* and the Japanese pearl-oyster, *Pinctada martensi,* in which ganglion cells were abundant near the A-V region, forming a ring at the most narrow portion of the heart. Krijgsman and Divaris (1955) stated that muscle fibers of the molluscan heart have two fundamental properties: automaticity (possess intrinsic beat independent of nerve supply) and contractility (ability of fibers to shorten). Ebara (1967, 1969) conclud-

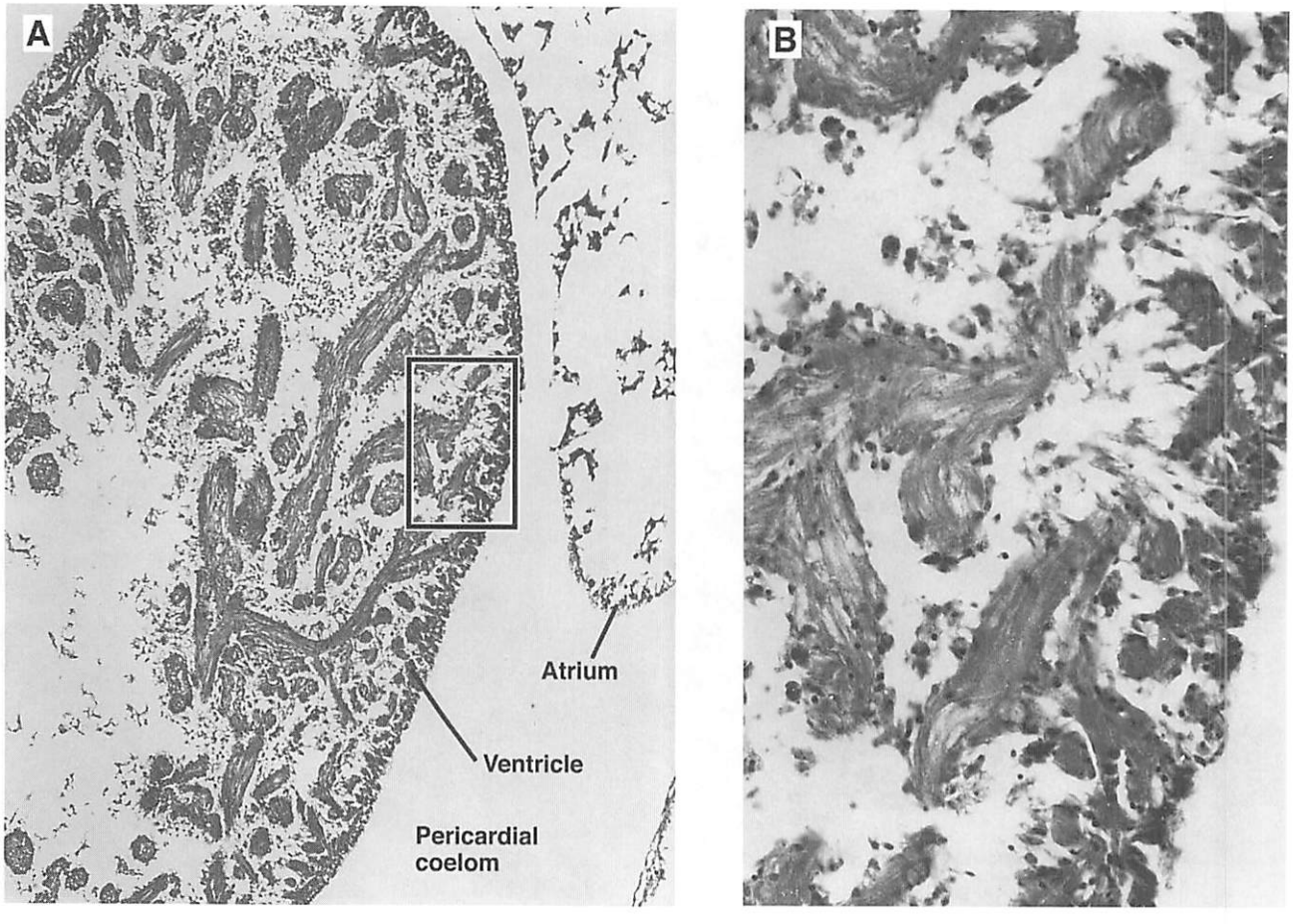


Figure 8. (A) Histological section of the ventricle near the atrium. HFW = 940 μm. (B) An enlargement of rectangle in (A) to show details of the myocardium. HFW = 224 μm.

ed that the overall rhythm of the oyster ventricle is formed as the result of functional interaction among myocardial fibers. Further, Ebara and Kuwasawa (1975) reported that all muscle fibers of the ventricle in *C. gigas* could be considered to possess automaticity and that the initiating site of cardiac rhythm in the excised ventricle was not fixed in a definite area but occasionally wandered from one locus to another, a so-called diffuse pacemaker (Irisawa 1978; Jones 1983). However, Ebara and Kuwasawa (1975) concluded that spontaneous contraction started from the region of the A-V boundary in intact ventricles. Thus, it appears that any area of the ventricular myocardium can act as a pacemaker (generally defined as cells that increase their rate of firing when subjected to transmembrane pulses of depolarizing current and slow the rate when hyperpolarized [McCann 1970]). In the intact ventricle, however, the functional pacemaker activity seems to coincide with the concentration of ganglion cells near the base of the ventricle at the A-V boundary. For further details, see Jones (1983) who reviewed cardioregulation in bivalves and discussed myogenicity and the pacemaker, heart action potentials including EKGs, and innervation and neural control.

Uesaka et al. (1987a, b) discussed how the atria and ventricle coordinate rhythmical activities in *C. gigas*. In several different preparations the excised heart seemed to exist in two different states: (1) the ventricle possessed the higher firing rate and the heart beat with a short A-V delay and (2) the atrium had the higher rate and the heart beat with a long A-V delay. Further, if either the atrium or ventricle for that specimen had the higher intrinsic rhythm, then that portion would accelerate the rhythm of the other; the one with the slower rhythm would, conversely, inhibit the rhythm of the other.

Uesaka et al. (1987b) found that atria and ventricles stimulated each other by mechanical stretching only and that myocardial bundles that might function as a conductile tissue did not cross the boundary of the A-V valve. If this stretching is prevented by either mechanical means or surgical separation of the atria from the ventricle, each portion of the heart establishes its own rhythm independent of the other. When stretching between atria and ventricle is reestablished, the normal rhythm is restored. If the stretch is applied during the falling phase of the action potential, it causes prolongation of the beat interval compared to the preceding control interval; conversely, stretch applied between maximum hyperpolarization and the peak of the next action potential causes shortening of the beat interval and increases the rate of rise of the pacemaker (action) potential. These results indicate that stretching may induce depolarization of the membrane potential of myocardial cells.

Hevert (1984) measured intraventricular pressures in *C. gigas*; systolic pressure averaged 32.5 ± 7.0 mm H_2O (n=23) and diastolic pressure averaged 3.0 ± 2.0 mm H_2O (n=23). This systolic pressure was almost twice that reported by Smith and Davis (1965) for *C. gigas* (19.0 mm H_2O) but seems to be about average for bivalve ventricular pressures tabulated by Jones (1983).

Hevert (1984) noted that pericardial pressures oscillated between –3.0 mm H_2O (ventricular systole) and +3.0 mm H_2O (ventricular diastole). These low pericardial pressures during ventricular systole are probably important in helping to fill the atria from veins where pressures are very low (Ramsay 1952).

Consistent with other studies, Hevert (1984) found *C. gigas* to be an osmotic conformer over the range 902 to 1358 mM kg^{-1}; pericardial fluids were not significantly different from hemolymph over this range. Shumway in Chapter 13 provides a detailed discussion of osmoregulation in *C. virginica*. Large differences were found between hemolymph and pericardial fluids (Hevert 1984) for colloid osmotic pressure and protein content in *C. gigas* (Table 1).

Hevert (1984) calculated an effective filtration pressure from known values of hydrostatic pressures and colloidal osmotic pressures of the heart and pericardial fluid. At systole, the effective filtration pressure for *C. gigas* was 31.7 mm H_2O from the heart into the pericardial coelom and at diastole it was 3.8 mm H_2O in the reverse direction (Fig. 9). Hevert (1984) estimated that about ten times as much fluid flows out of the heart as flows back during each heart beat. Using inulin, he determined the rate of ultrafiltration (the net loss of low molecular weight hemolymph

constituents from the heart to the pericardial coelomic fluid) in *C. gigas* to be 0.4 L g^{-1} min^{-1} which corresponded to about 23 ml d^{-1} for the whole animal. This fluid collects in the pericardial coelom and moves into the kidney via the reno-pericardial canal where a small portion is eliminated as urine; the bulk of the fluid is reabsorbed into the renal sinuses and returned to the circulatory system. Eble in Chapter 2 discusses details of the excretory system

Wendt and Hevert (1985) demonstrated that Ca^{2+} at physiological concentrations was transported from the pericardial fluid into the ventricle of *C. gigas* via Ca^{2+} channels that were sensitive to verapamil, a calcium channel blocker. This drug caused a significant decrease in the transport of Ca^{2+} into the ventricle but because it did not completely block the transport. Wendt and Hevert (1985) concluded that more than one type of calcium channel must exist.

Wendt (1987) showed that the ventricle of *C. gigas* actively transported Mg^{2+} into the pericardial fluid and that the transport could be blocked by 2,4-dinitrophenol (a drug that uncouples phosphorylation from oxidation in mitochondria). Wendt (1987) also concluded that the Mg^{2+} transport was not Ca^{2+} dependent.

Different ions have different effects on the intact and isolated heart of oysters. Otis (1942) found that a decrease in pH from 8 to 4 slowed the beat of the isolated heart of *C. virginica* in a perfusion chamber; below pH 4 the heart ceased beating. On the other hand, heart rate steadily increased as pH rose from 5 to 9, becoming irregular at pH 9 and stopping in systole at pH 10.

Isoosmotic potassium and sodium stopped heart beat of *C. virginica* in perfusion chambers in systole whereas isoosmotic calcium stopped heart beat in diastole (Otis 1942). Normal activity was quickly restored when hearts were returned to van't Hoff solution, showing that the oyster heart, similar to all other animal hearts studied, beats normally only in balanced salt solutions. Perrine et al. (1971) studied the effects of potassium- and sodium-enriched solutions on heart rate, amplitude of beat, cardiac work, and lethal temperatures on the isolated ventricles of *C. angulata*. They found that Ca^{2+} increased the amplitude of contraction relative to the reference solution whereas K^+ increased the number of contractions as well as cardiac work (the sum of the amplitudes, expressed in millimeters, recorded in 200 seconds) relative to the reference solution at 32°C.

The chemical control of the bivalve heart has been reviewed by Krijgsman and Divaris (1955), Greenberg (1965), Hill and Welsh (1966), Welsh (1971), Greenberg et al. (1980), and Jones (1983). Three (and perhaps four) substances are accepted as physiologically active molluscan neurotransmitters:

Table 1. Differences in attributes between hemolymph and pericardial fluid in *Crassostrea gigas* (from Hevert 1984). Mean ± SD where indicated. N = number of samples.

Attribute	Hemolymph	Pericardial Fluid
Colloid osmotic pressure (mmH_2O)	4.3±1.9 N = 27	0.5±0.9 N = 27
Protein content ($g\ L^{-1}$)	2.4±0.08 N = 9	0.09±0.08 N = 11
Average protein molecular weight (Daltons)	141,000	45,000
Protein concentration ($\mu m\ L^{-1}$)	17	2

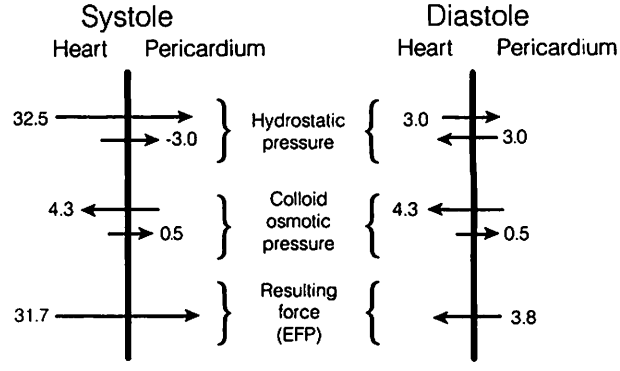


Figure 9. Compilation of all physical forces driving fluid through the heart wall of *Crassostrea gigas*. Arrows indicate direction of resulting flow; pressures are recorded in mm H_2O. EFP [effective filtration pressure]. From Hevert (1984).

acetylcholine (ACh), 5-hydroxytryptamine, and phenylalanyl-methionylarginylphenylalanine amide (FMRFamide); dopamine is a fourth possibility (see Jones [1983] for a detailed review).

Elliott (1980) reported that the response of the isolated *C. virginica* heart to acetylcholine chloride (ACh) was usually biphasic with a relatively rapid but weak depolarization immediately followed by a prolonged hyperpolarization. In contrast, in the hard clam, *Mercenaria mercenaria,* there was a hyperpolarization that was slow in developing but long in duration whereas the isolated ventricle of the blue mussel, *Mytilus edulis,* responded with a rapid depolarization. Elliott (1980) stated that the ionic mechanisms responsible for the hyperpolarizing phase, at least in *M. mercenaria* and *C. virginica* were due solely to a K^+ permeability increase and that this response could be blocked preferentially by methylxylocholine but not by tubocurarine or hexa-methonium. She also showed that the rapid depolarizing phase of the ACh response of the isolated heart of *C. virginica* was due solely to an increase in Cl^- permeability that could be blocked preferentially by tubocurarine; hexamethonium and methylxylocholine were not very effective in blocking this response (Elliott 1980).

Elliott (1980) concluded that ACh is the transmitter of inhibitory motor neurons in molluscs such as *Aplysia* sp. as well as in the bivalve families Ostreidae and Veneridae. Further, she reasoned that the physiological role of ACh in these hearts was to cause inhibition by an increase in K^+ permeability. She was convinced that the multiphasic responses of oyster heart cells to ACh was due to the presence of more than one type of ACh receptor in the population of heart cells.

The Aortae

The ventricle gives rise to the anterior and posterior aortae at the region of its most dorsal extremity (Fig. 4A). The posterior aorta leaves the dorsoposterior portion of the ventricle and runs posteriorly immediately under the rectum to which it is attached (Fig. 4A). As it passes along the ventral surface of the rectum it gives off rectal arteries (Figs. 4A and 10). When it reaches the dorsoanterior corner of the adductor muscle it turns abruptly ventral and runs down the anterior medial surface of the muscle; midway, it takes another right angle turn into the tissues of the muscle where it divides into the numerous arteries that supply this vascular organ (Fig. 4A). The eastern oyster, unlike many bivalves, especially those with siphons, lacks a posterior aortic bulb; its function is to act as a temporary reservoir for hemolymph squeezed out of the siphon and the posterior adductor muscle when these organs contract. When the adductor muscle of the eastern oyster contracts, hemolymph is forced under pressure back into the ventricle which suddenly dilates to accommodate this surge. Were it not for the fluid in the pericardial coelom, the ventricle would rupture under the force of this pressure.

The anterior aorta leaves the anterodorsal portion of the ventricle and enters the visceral mass accompanied by a dorsal extension of the pericardial

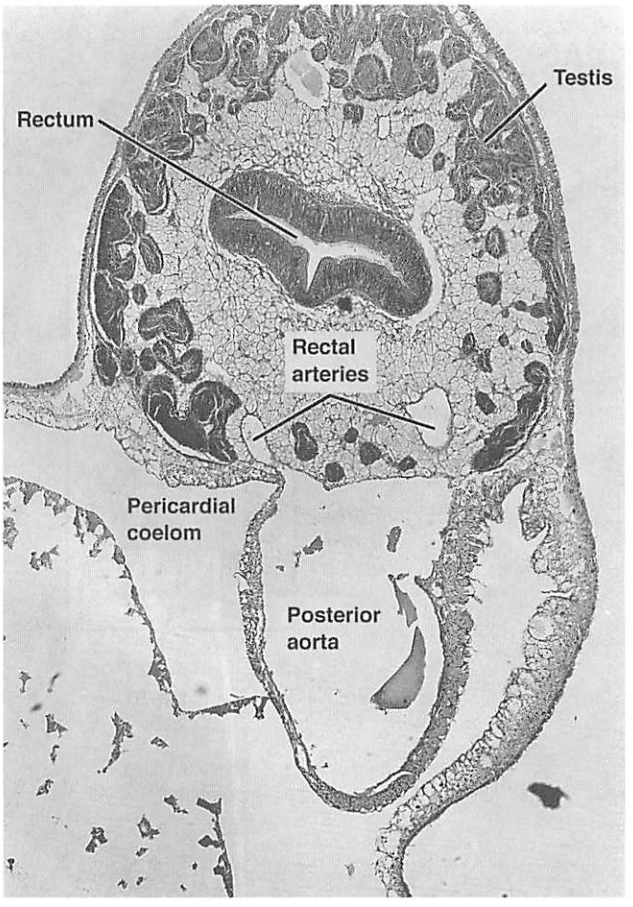


Figure 10. Transverse section through rectum to show the posterior aorta giving rise to rectal arteries. HFW = 1.5 mm.

coelom for a few millimeters (Fig. 11). A single semilunar valve is located at the entrance to the anterior aorta. This typical pocket valve consists of muscle tissue covered on both sides by a layer of cells similar in appearance to the endocardial-like cells of the ventricle and is attached at the blind end to the dorsal wall of the anterior aorta (Fig. 11).

CIRCULATORY SYSTEM

Visceral Mass

Only the main vessels of the arterial and venous systems of the visceral mass will be discussed. Figures 4A and 4B are composite interpretations of the arterial and venous systems. Circulation of hemolymph in gills, adductor muscle, kidney, and mantle are each discussed separately. Details of the entire circulatory system can be found in Eble (1963).

The Arterial System. About 1 to 2 mm from the ventricle, the anterior aorta enlarges to form the anteroaortic bulb from which several large arteries spring. Two large, posterior gastric arteries originate at the right, ventral side of the anteroaortic bulb and run in the pyloric process (ventral posterior extremity of the visceral mass [Galtsoff 1964]). These arteries give off numerous branches that form a basket-like meshwork of vessels that supply the entire length of the mid-gut and style sac (Fig. 12). Also arising from the anteroaortic bulb are two large arteries that sup-

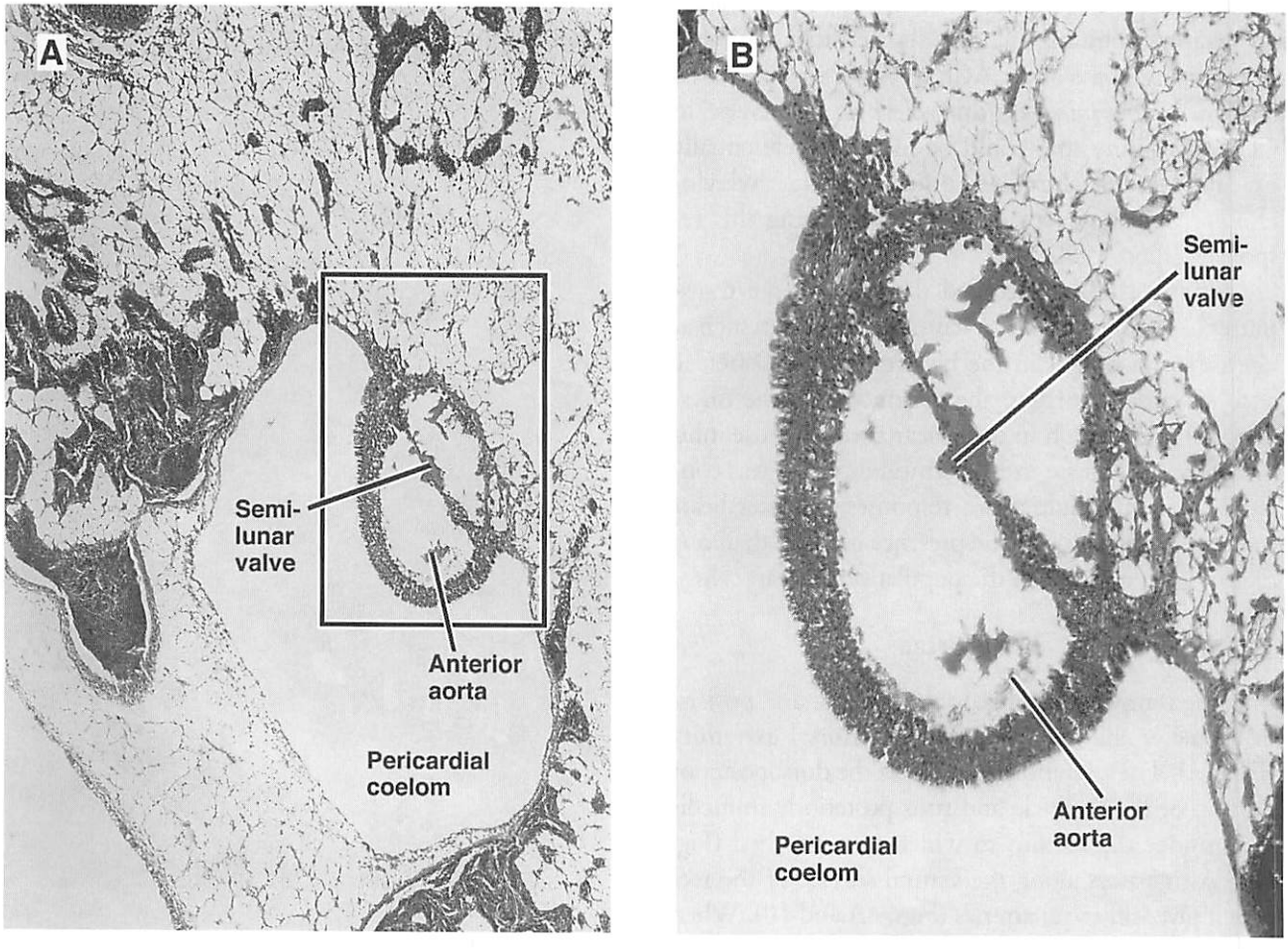


Figure 11. (A) Semilunar valve of the anterior aorta. HFW = 394 μm. (B) An enlargement of rectangle in (A). HFW = 130 μm.

ply the left posterior visceral mass; these are designated the ventral visceral and posterior left visceral arteries. The posterior pallial artery originates in the vicinity of the anteroaortic bulb and immediately runs out to supply the left posterior area of the mantle.

The anterior aorta gives off many small hepatic arteries to the digestive gland as it runs through the dorsal region of the visceral mass. Approximately in the middle of its course the anterior aorta gives off the large median visceral artery from its left side supplying the stomach, digestive gland, intestine, and gonad.

Just before the point of origin of the common labial artery, the anterior left visceral artery arises from the left side of the aorta to supply the anterior portions of the stomach, digestive gland, intestine and gonad. Arising either directly from the dorsal aorta or from the common labial artery is the prominent right visceral artery that supplies organs in the right side of the visceral mass.

The large common labial artery comes off the dorsal aorta and branches twice to form the four labial arteries that supply the labial palps. Almost immediately after giving off the common labial artery, the anterior aorta swings sharply to the left to supply the tissues of the oral hood. Upon reaching the extremity of the oral hood, the anterior aorta bifurcates into a short, dorsal, common circumpallial artery and a long, oral hood artery that runs in the margin of the oral hood. The oral hood artery continues in a ventral di-

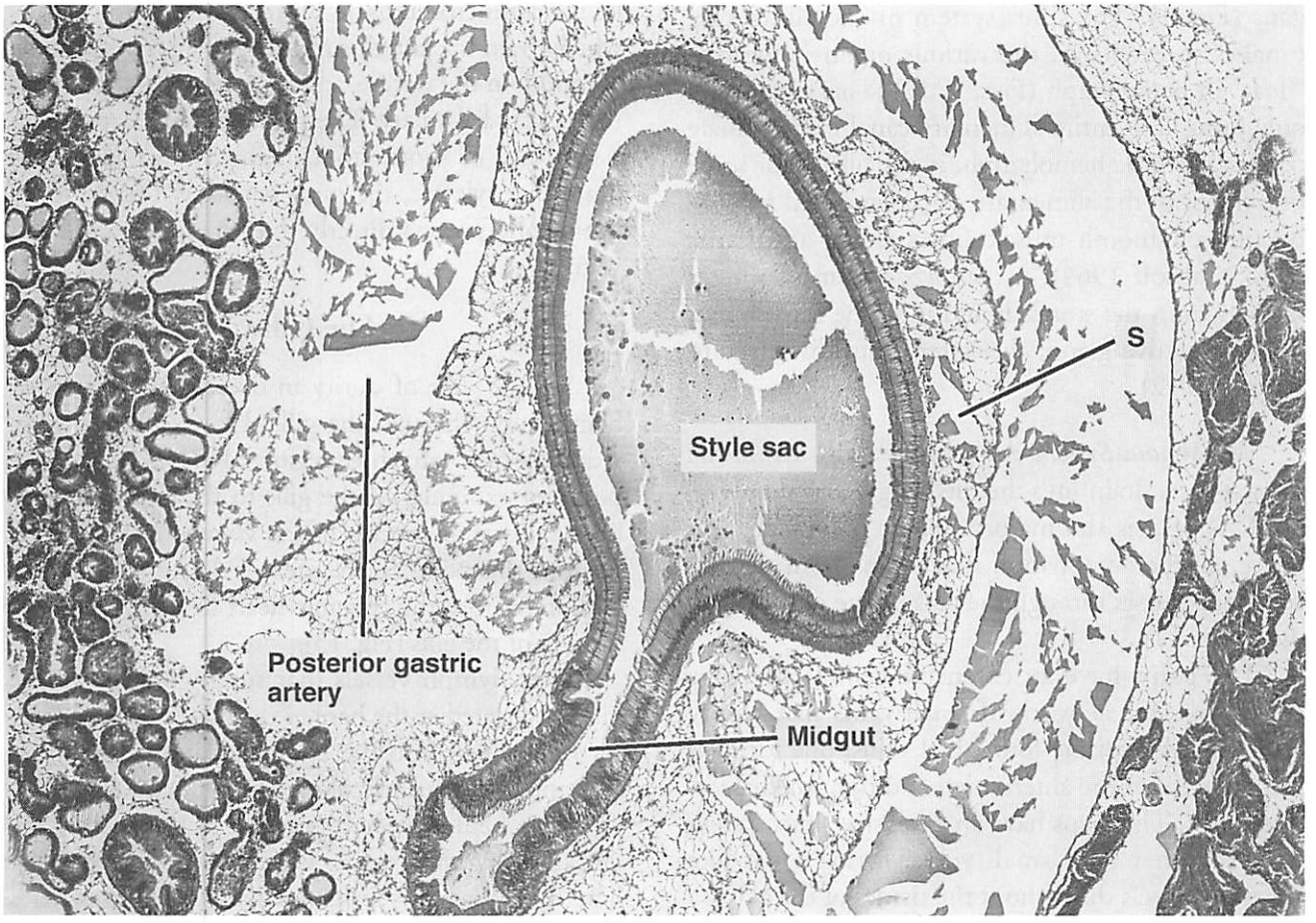


Figure 12. Transverse section through the pyloric process to show posterior gastric arteries supplying the style sac and midgut. [S] hemolymph sinus. HFW = 1.5 mm.

rection to the point where the left and right ventral mantle lobes bifurcate from the oral hood; here it divides into the right and left ventral circumpallial arteries that run in the mantle margins.

The common dorsal circumpallial artery also divides into right and left dorsal circumpallial arteries at the point where the dorsal left and right mantle lobes bifurcate from the oral hood. These arteries deliver blood to the tentacles of the pallial margins and to the sinuses of the mantle. Branching from the oral hood artery are the anterior pallial arteries that run posteriorly in both mantle lobes for considerable distances before ending in pallial sinuses; they branch profusely and can be easily seen as translucent vessels in the living oyster.

The arterial wall gradually disappears as the artery merges with sinuses that bathe the visceral organs (Fig. 1). The sinus system of the alimentary canal is so extensive, the various organs appear to "float" in hemolymph (Figs. 1, 12). This system not only brings the entire alimentary canal into intimate contact with the hemolymph, it also allows for some movement of the alimentary canal generated by contraction of smooth muscle fibers (Shaw and Battle 1957; Galtsoff 1964). A complex system of sinuses radiates from the stomach and intestine into sinuses of the digestive gland; the latter drain directly into veins (Fig. 12).

The Venous System. All veins (see Fig. 4B) of the visceral mass drain into the medial gill axis vein (Fig. 2). This vein is the major afferent branchial vein; hence, all the hemolymph that drains from the visceral mass passes through the gills before returning to the heart.

The palps have an extensive venous drainage consisting of a network of small veins that empty into a labial vein; the four labial veins, one for each palp, drain into the anterior left and right medial visceral veins. The palps have an extensive sinus system that, together with small veins, forms a net-like plexus of vessels throughout the tissues of the palps.

The anterior medial visceral veins drain the anterior extremities of the visceral mass and palps. They originate in the dorsoanterior region of the visceral mass and run in a ventroposterior direction, roughly following the line of insertion of the palps with the visceral mass. After receiving the labial veins. They turn medially and fuse in the mid-ventral line to form the large medial gill axis vein which, as the name implies, runs in the axis between the two gills.

The recto-visceral vein is the largest vein in the visceral mass. It originates in the dorsal region of the visceral mass, then swings ventrally near the pericardial cavity where it receives the large rectal vein. The recto-visceral vein then runs superficially in the left side of the visceral mass from where it receives many veins that drain this posterior region and finally empties into the medial gill axis vein.

The right and left viscero-adductor veins drain the region just anterior to the pericardial cavity. They also receive large branches from the anterior portion of the adductor muscle (anterior adductor veins), which drain the anterior region of the adductor muscle and join the viscero-adductor veins just ventral to the pericardial cavity. The posterior gastric veins, an assemblage of short, stout, highly branched vessels, drain the style sac, the midgut and all the tissues in the pyloric process directly into the median gill axis vein.

The Gills

For the sake of clarity in discussing the flow of hemolymph through the gills, I have arbitrarily divided the gills into three regions: Region 1: from the posterior extremity of the gills to the area of the tip of the posterior process of the visceral mass; Region 2: from this point to the area of the systemic heart; Region 3: from the systemic heart to the anterior extremity of the gills (Fig. 13).

Hemolymph vessels that supply and drain the gills are located at the base of the gills (where the water tubes open into the epibranchial chamber) and run antero-posteriorly along the gill axes (Figs. 14, 15). Five hemolymph vessels run in the base of the gills, deriving their names from the axes they occupy. The vessel that runs in the median axis that separates the two gills is the median gill axis vein and is the major afferent branchial vein (Fig. 15). In the axis between the two demibranchs of each gill there lies a

smaller vessel, the lateral gill axis vein, which is continuous only in the anterior region of the gills (Fig. 14) and serves to drain them of hemolymph. In the posterior region of the gills, this vessel is subdivided into many short, discontinuous vessels that lie in the same axis but which drain into the pallio-branchial vein (Figs. 14, 15). This latter vein lies in the confluence between the gill and mantle and drains hemolymph from the gill and mantle and returns it directly to the heart (Figs. 14, 15, 16).

Region 1. The hemolymph supply to the gills in this region is exclusively by the medial gill axis vein which constitutes the afferent branchial vessel. Hemolymph flows in the medial gill axis vein in a posterior direction. Hemolymph vessels spring from this vein to every other principal filament of the inner lamella of the inner demibranch. Hemolymph is, in addition, supplied indirectly via the vascular interlamellar junctions to the opposing principal filaments of this same demibranch (Figs. 14, 15). It is also supplied directly to every other principal filament of the outer lamella of the outer demibranch by means of vessels that originate from the medial gill axis vein and run across both interlamellar junctions at the gill base to the hemolymph vessels of these principal filaments (Fig. 15). Hemolymph vessels of the opposing principal filaments in the inner lamella of the outer demibranch receive their he-

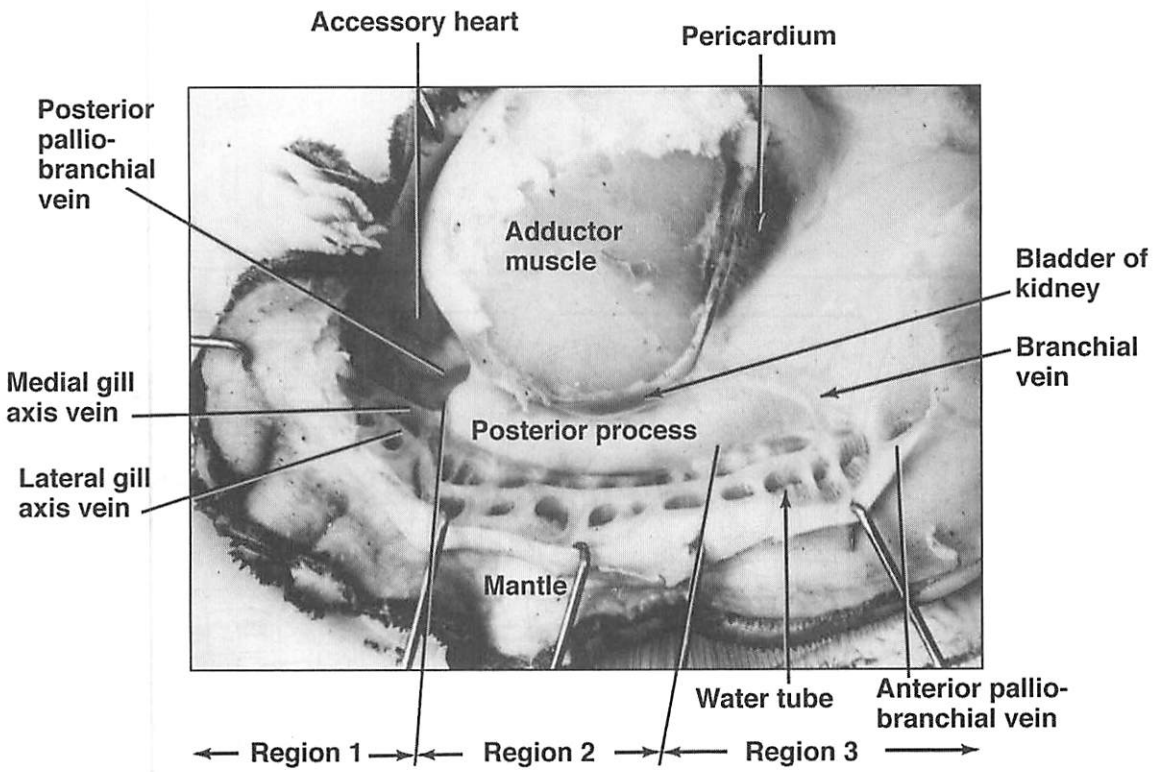
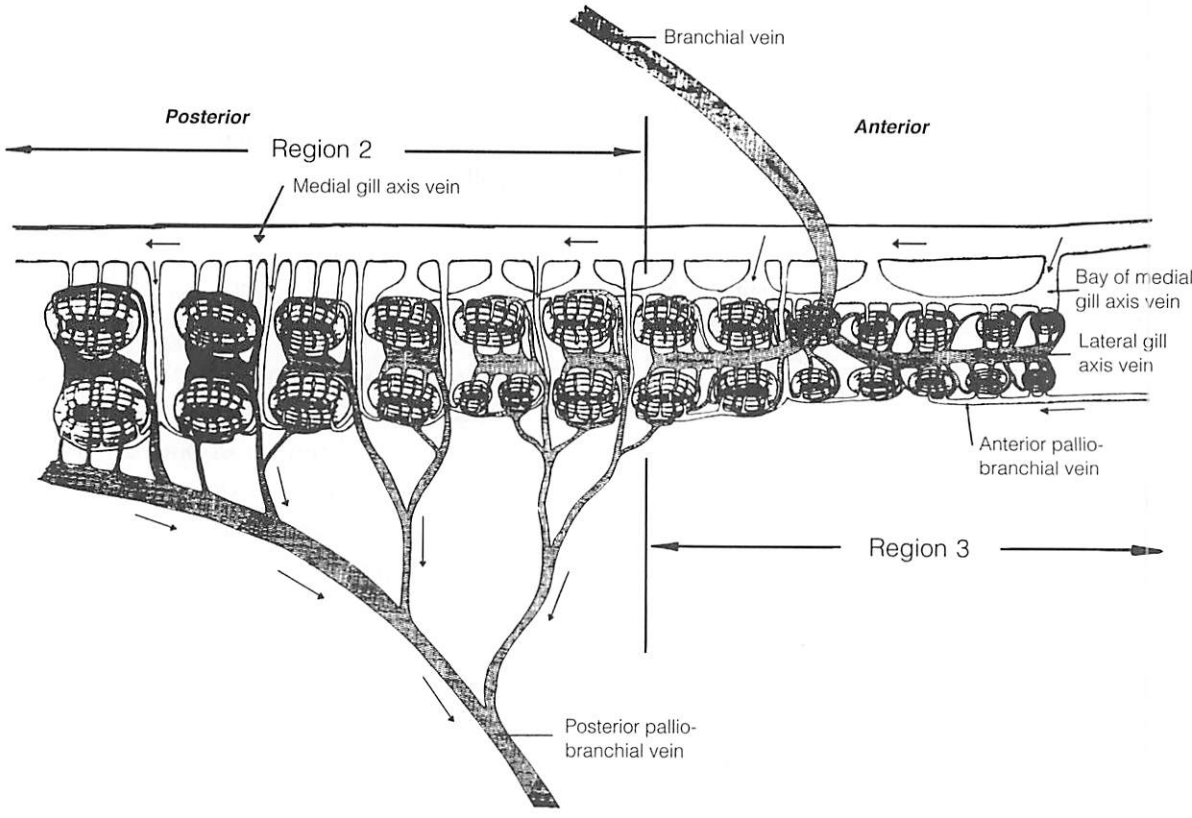
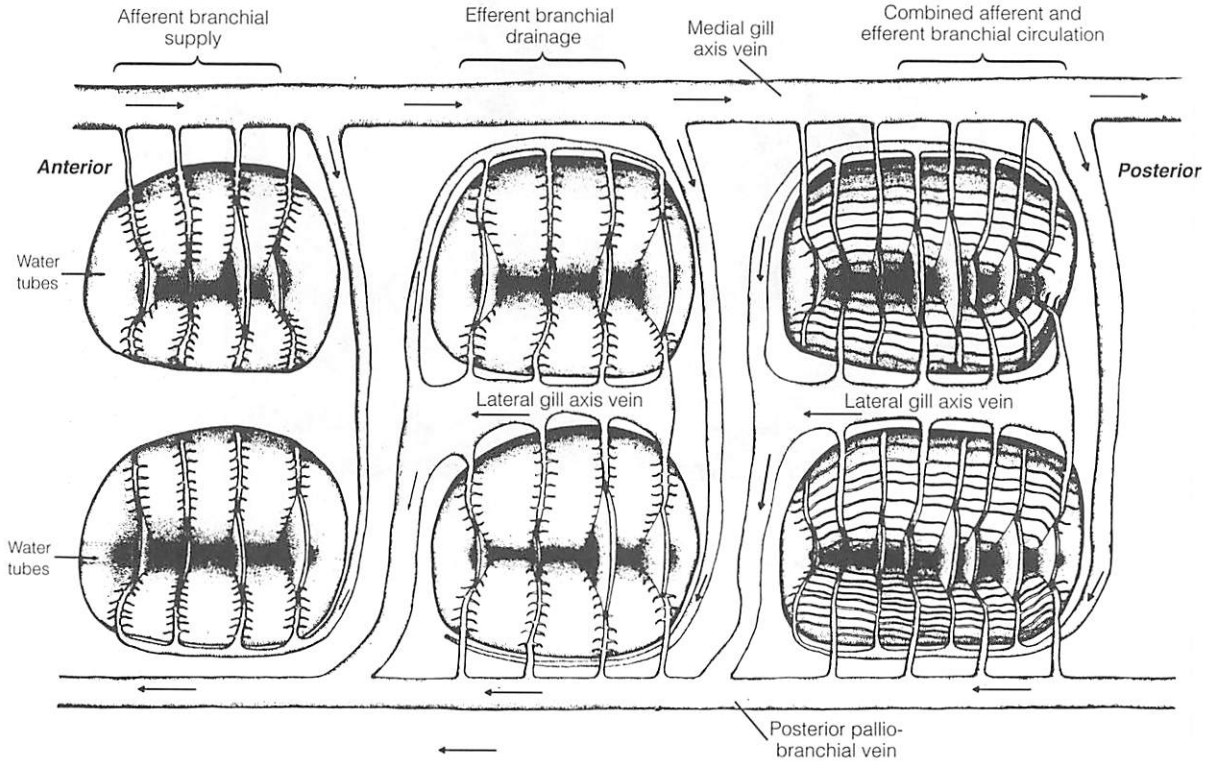


Figure 13. Photograph of right posterior view of an eastern oyster (× 3); the dorsal portion of the mantle has been removed, exposing the epibranchial chamber. The ventral area has been arbitrarily divided into: Region 1; Region 2; Region 3 (see text). Photograph taken by Dr. T.C. Nelson.


Branchial vein
Posterior
Anterior
Region 2
Medial gill axis vein
Bay of medial gill axis vein
Lateral gill axis vein
Anterior pallio-branchial vein
Region 3
Posterior pallio-branchial vein


Afferent branchial supply
Efferent branchial drainage
Medial gill axis vein
Combined afferent and efferent branchial circulation
Anterior
Posterior
Water tubes
Water tubes
Lateral gill axis vein
Lateral gill axis vein
Posterior pallio-branchial vein

molymph supply indirectly via the vascular interlamellar junctions.

Principal filaments of the efferent branchial system alternate with those of the afferent branchial system such that adjacent principal filaments of a lamella transport hemolymph in different directions (Fig. 17). The lateral gill axis vein drains all efferent principal filaments with the exception of those of the outer lamella of the outer demibranch. The lateral gill axis vein runs distances of only one or two water tubes before turning laterally to join the pallio-branchial vein (Fig. 15). Efferent principal filaments of the outer lamella of the outer demibranch join directly with the pallio-branchial vein; this vessel, in addition to being the major efferent branchial vessel in this region, drains the broad face of the mantle before returning hemolymph to the heart (Fig. 16).

Region 2. The afferent branchial supply in Region 2 is identical to that described for Region 1 with two minor variations. First, the medial gill axis vein usually curves dorsally as it enters the visceral mass in the anterior region of the oyster. Because this necessitates longer and longer connections to the afferent principal filaments, the medial gill axis vein extends ventrally as a regular series of small bays from which vessels lead to afferent principal filaments (Fig. 14); second, branches from the medial gill axis vein that supply the outer lamellae of the outer demibranchs frequently bifurcate at their lateral extremity to supply afferent principal filaments of two adjacent water tubes (Fig. 14).

The major features of the efferent branchial system are also similar to those described for Region 1, with minor differences due to the fact that the pallio-branchial vein moves away from the gills as it ascends to join the systemic heart. The short segments of the lateral gill axis vein must, of necessity, gradually lengthen in the area near the heart to join with the pallio-branchial vessel. The efferent principal filaments of the outermost lamellae of the gills also lengthen and usually many of these small vessels coalesce into a common vein before emptying into the pallio-branchial vein (Fig. 14).

Region 3. The important feature of this region is that the lateral gill axis vein becomes a continuous

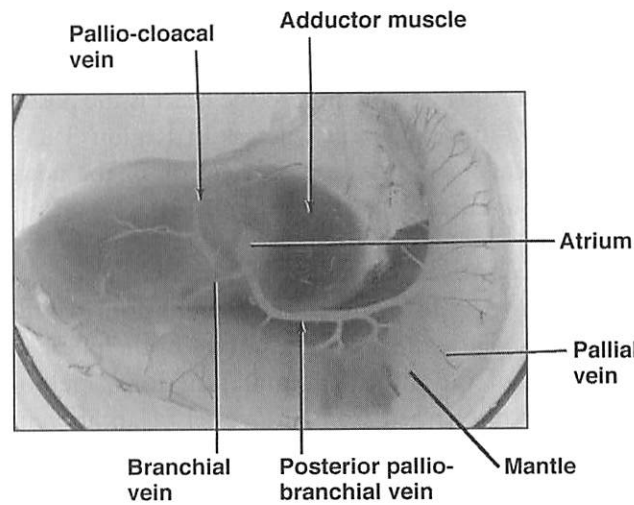


Figure 16. Whole mount of oyster cleared with cedar wood oil, viewed from left side (× 0.5). The atrium was injected with red vinyl acetate which backed up into the great veins that return blood to the heart.

Figure 14 (opposite page top). Composite drawing of the anterior branchial hemolymph circulation (Regions 2 and 3) viewed from the right side (× 7). Afferent branchial supply — clear; efferent branchial drainage – shaded. Arrows indicate the path of hemolymph. Note sinus-like bays of the medial gill axis vein which lead directly into the hemolymph vessels of the afferent principal filaments of the innermost lamellae of both gills; mantle removed to expose gill base.

Figure 15 (opposite page bottom). Schematic drawing of the afferent and efferent circulation of the left gill in Region I seen from its base (× 27). The circulatory patterns of the corresponding region of the right gill are the same as the left; the anterior portion of the figure is on the left and the mantle has been stretched away from the body in order to dilate the water tubes. The afferent and efferent hemolymph vessels have been isolated in the left and middle pair of water tubes, respectively. The composite afferent and efferent circulation is represented at the right of the figure. The direction of hemolymph flow is represented by the arrows.

vessel and returns hemolymph directly to the heart as the branchial vein. The major afferent supply to the gills in this region is via the medial gill axis vein, as in Regions 1 and 2. In the anterior extremity of the gills, however, a new afferent branchial vessel arises, the anterior pallio-branchial vein (Fig. 14), that supplies hemolymph to the afferent principal filaments of the outermost lamellae of the gills and drains the lateral palps and adjacent regions of the mantle (Fig.14).

The efferent branchial circulation of Region 3 is essentially the same as that in Regions 1 and 2. However, the lateral gill axis vein returns hemolymph directly to the heart instead of merging with the pallio-branchial vein; it ascends to the heart as the branchial vein in the septum that partitions the epibranchial space into two separate chambers (Fig. 14).

Thus, in Regions 1 and 2 the efferent branchial vessel returning hemolymph to the heart is the posterior pallio-branchial vein whereas in Region 3 it is the branchial vein (the dorsal extension of the now-continuous lateral gill axis vein [Fig. 14]).

Plical Sinuses. The normal pathway from the afferent principal filaments to the efferent principal filaments is via the hemolymph sinuses of the plicae at the level of the interfilamentar junctions. Hemolymph is discharged from the vessel of an afferent principal filament into the plical sinus which, in turn, opens into the hemolymph vessels of the ordinary filaments (Fig. 17). These all open into the plical sinus at every interfilamentar junction. Hemolymph vessels of ordinary filaments are not patent from base to apex of the gill, because at every interfilamentar junction the hemolymph vessel is obliterated by a "baffle" system which consists of skeletal rods of ordinary filaments that elongate to serve as points of attachment for interfilamentar muscles (Fig. 18). Thus, hemolymph is forced out from the vessel of the ordinary filament into the plical sinus at every interfilamentar junction and this, coupled with the fact that afferent and efferent principal filaments usually connect only with every other plical sinus, ensures that hemolymph flows efficiently from afferent to efferent branchial systems (Fig. 17).

Ordinary filaments have large hemolymph sinuses that are spanned by lacunar cells (Fig. 19). These cells are narrow, attach to epithelial cells of the filament by slender processes, and have a faintly eosinophilic cytoplasm and a spherical, centrally placed nucleus. The irregular spacing of lacunar cells gives the hemolymph sinus a cobweb-like appearance (Fig. 20).

The Adductor Muscle

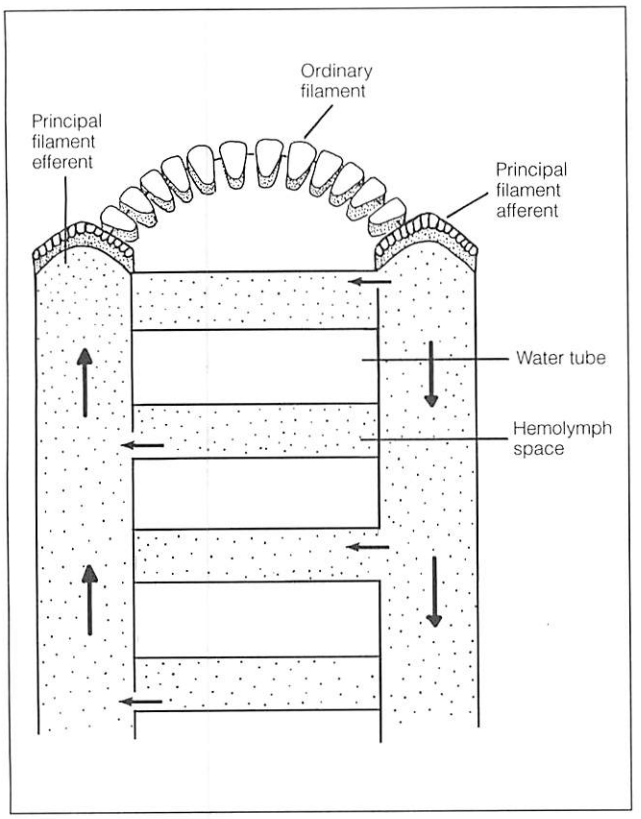
The adductor muscle is supplied with hemolymph by the posterior aorta. As soon as this vessel enters the muscle it divides into several large arteries; two trunks usually course laterally while one or two continue to run in the median line. Each of these major arteries then proceeds to subdivide into many branches that take a very irregular course in the mus-

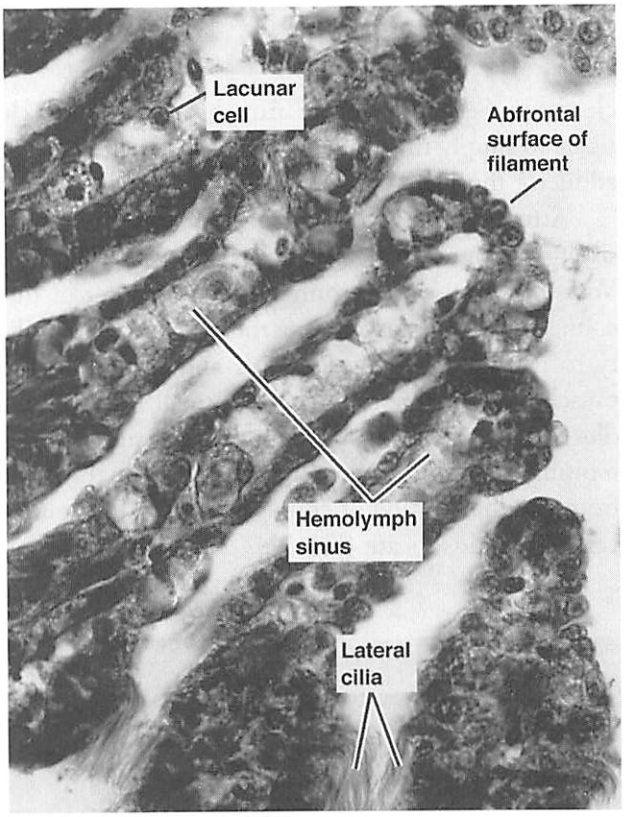
Figure 17 (top left). Diagrammatic sketch of a plica viewed from its medial surface, i.e., the side that faces the water tube (× 30). The afferent principal filament is on the right; the efferent principal filament is on the left. The arrows indicate the direction of hemolymph flow. The drawing is accurately scaled to let 10 μm equal 5 mm; the entire drawing represents a continuous series of approximately 1200 μm proceeding basoapically. The clear areas represent the "water space" (the large channels through which the water flows from ostia to water tube).

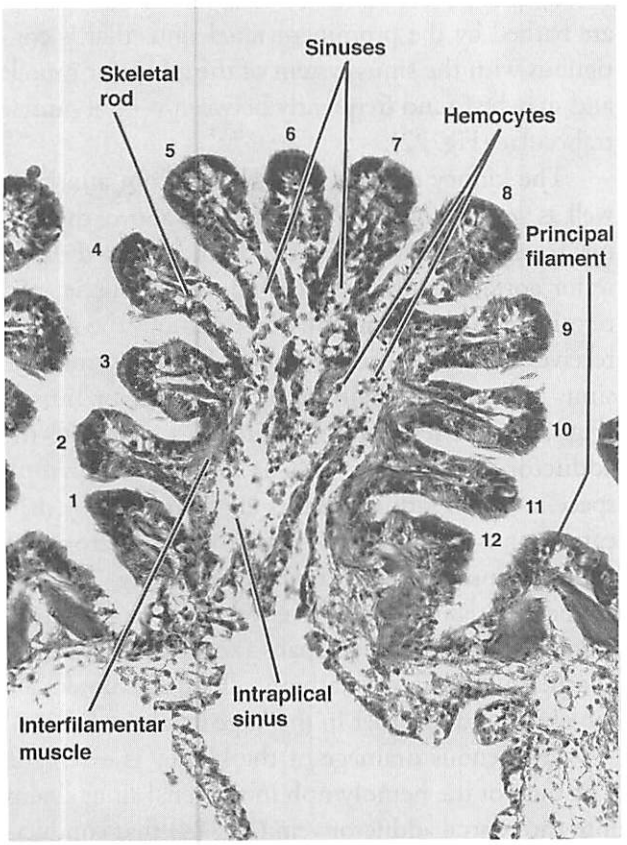
Figure 18 (bottom left). Frontal section of gill at level of an intraplical sinus. HFW = 305 μm. Note how skeletal rods elongate in Filaments 1, 2, 3, 9, 10, 11, 12 to receive insertion of the interfilamentar muscle, thus obliterating the hemolymph sinuses of these filaments. Hemolymph sinuses of Filaments 4 to 8 are still patent due to the oblique section.

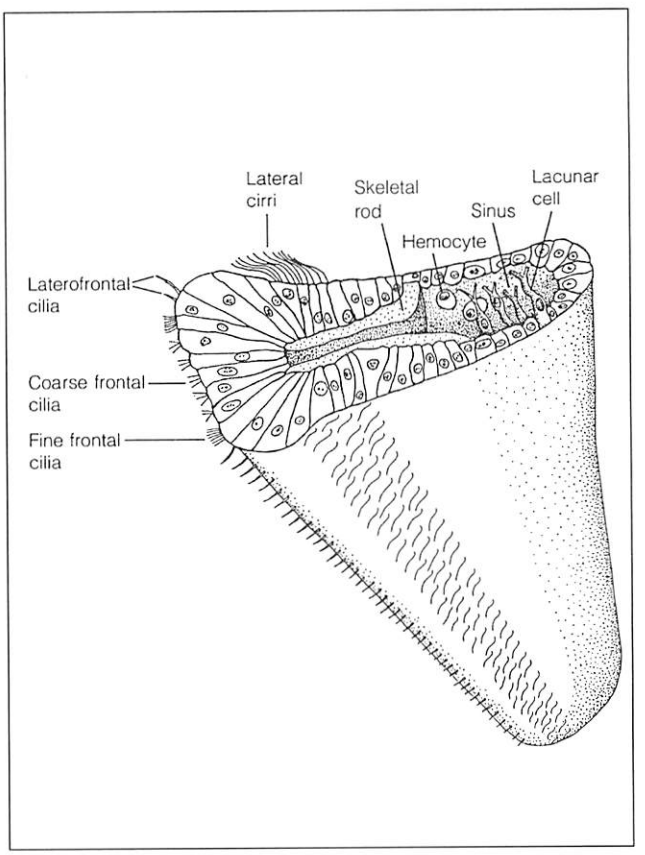
Figure 19 (top right). Transverse section through ordinary filaments to show large hemolymph sinuses. HFW = 78 μm.

Figure 20 (bottom right). Stereodiagram of an ordinary filament illustrating the cobweb-like arrangement of lacunar cells as they span the hemolymph sinus of the filament (× 38).


Ordinary filament
Principal filament efferent
Principal filament afferent
Water tube
Hemolymph space


Lacunar cell
Abfrontal surface of filament
Hemolymph sinus
Lateral cilia


Sinuses
Skeletal rod
Hemocytes
Principal filament
1
2
3
4
5
6
7
8
9
10
11
12
Intraplical sinus
Interfilamentar muscle


Lateral cirri
Skeletal rod
Sinus
Lacunar cell
Hemocyte
Laterofrontal cilia
Coarse frontal cilia
Fine frontal cilia

cle tissue. Several branches run through the muscle tissue to supply the kidneys located just ventral to the adductor muscle.

Arteries in the adductor muscle have a lining consisting of connective tissue fibers and cells (Fig. 21). Veins and sinuses have only a very thin layer of this connective tissue surrounding them; indeed, the ability to distinguish between veins and sinuses in the muscle is extremely difficult because all muscle bundles are lined with a thin connective tissue. Arteries terminate in sinuses where the arterial lining thins out from the thick lining of connective tissue to the thin venous or sinus sheath (Fig. 21).

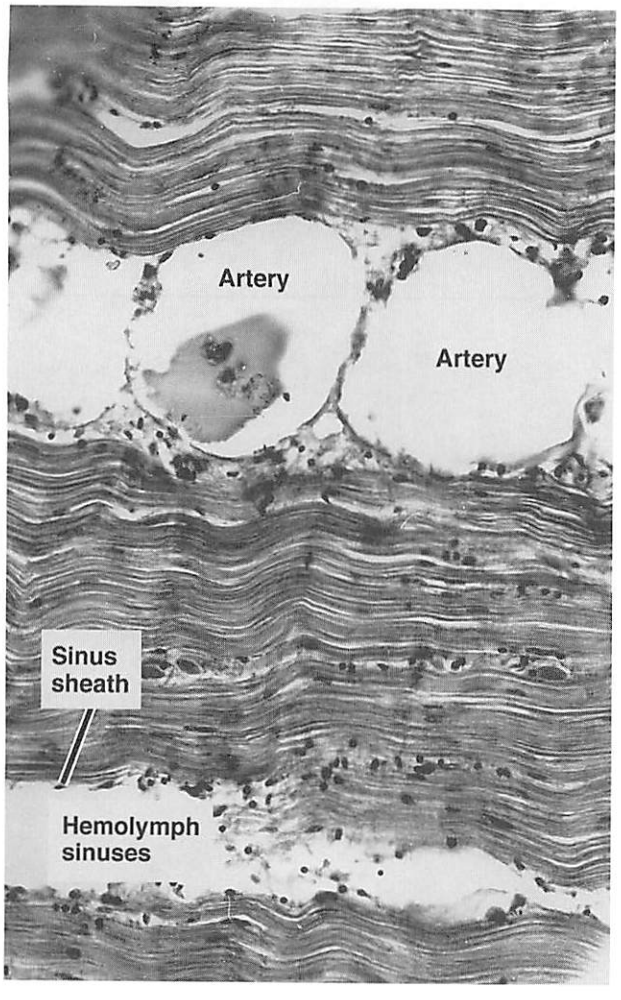


Figure 21. Transverse section through the adductor muscle showing arrangement of arteries and hemolymph sinuses. HFW = 190 μm.

The sinus-venous drainage of the adductor muscle begins in small clusters of anastomosing sinuses that run between and parallel to the muscle bundles (Fig. 22). These clusters of sinuses and small veins anastomose to form larger vessels, some of which run in a dorsoventral direction. All drain towards the gills and meet in the mid-ventral line to form the large renal sinus that bathes left and right kidneys (Fig. 22). The renal sinus drains into the large ventral adductor vein that runs along the left side of the pyloric process and empties into the median gill axis vein (Fig. 4B).

The adductor muscle has two other sites of venous drainage. The dorsal region of the adductor muscle, chiefly on its left side, is drained by the dorsal adductor vein that empties into the rectal branch of the recto-visceral vein. The anteroventral corners of the muscle are drained by the anterior adductor veins that empty into the viscero-adductor veins (Fig. 4B).

The Kidney

The kidney is a compound, tubular gland located adjacent to the adductor muscle. Kidney tubules are bathed by the prominent renal sinus that is contiguous with the sinus system of the adductor muscle and may be found frequently between ventral muscle trabeculae (Fig. 22).

The kidney receives hemolymph from arterial as well as venous sources. As mentioned above, the kidney receives several arteries that branch from the posterior aorta, run through the adductor muscle, and terminate in the renal sinus. In addition, the kidney receives the bulk of its hemolymph supply from the sinus and venous drainage of the adductor muscle (Fig. 22). The renal sinus, located just ventral to the adductor muscle (Fig. 22), is a large hemolymph space in which kidney tubules are suspended by delicate strands of connective tissue that run from the adductor muscle to the kidney tubules (Fig. 23). The connective tissue appears to envelope the kidney tubule as a thin sheet, then spans the sinus to attach to another tubule; in this fashion, all kidney tubules are loosely bound together in the large renal sinus.

The venous drainage of the kidney is extensive. The bulk of the hemolymph in the renal sinus drains into the ventral adductor vein (Fig. 24) that conducts

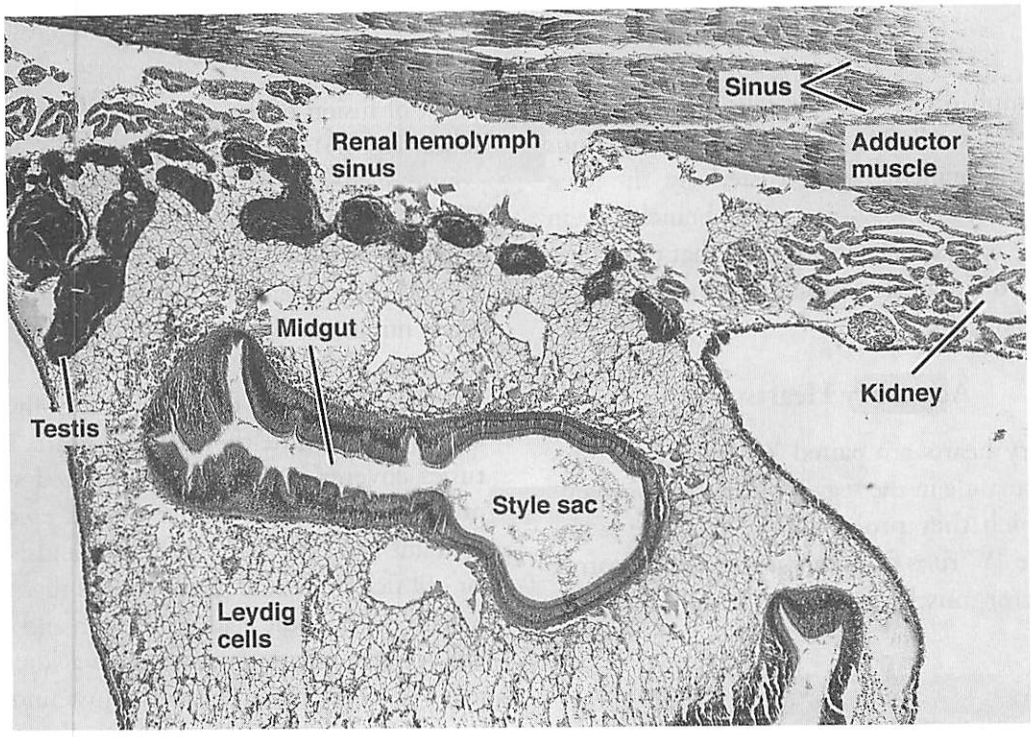


Figure 22. Transverse section through the kidney and adductor muscle. HFW = 2mm. Note large renal hemolymph sinus that is contiguous with hemolymph sinuses of the adductor muscle.

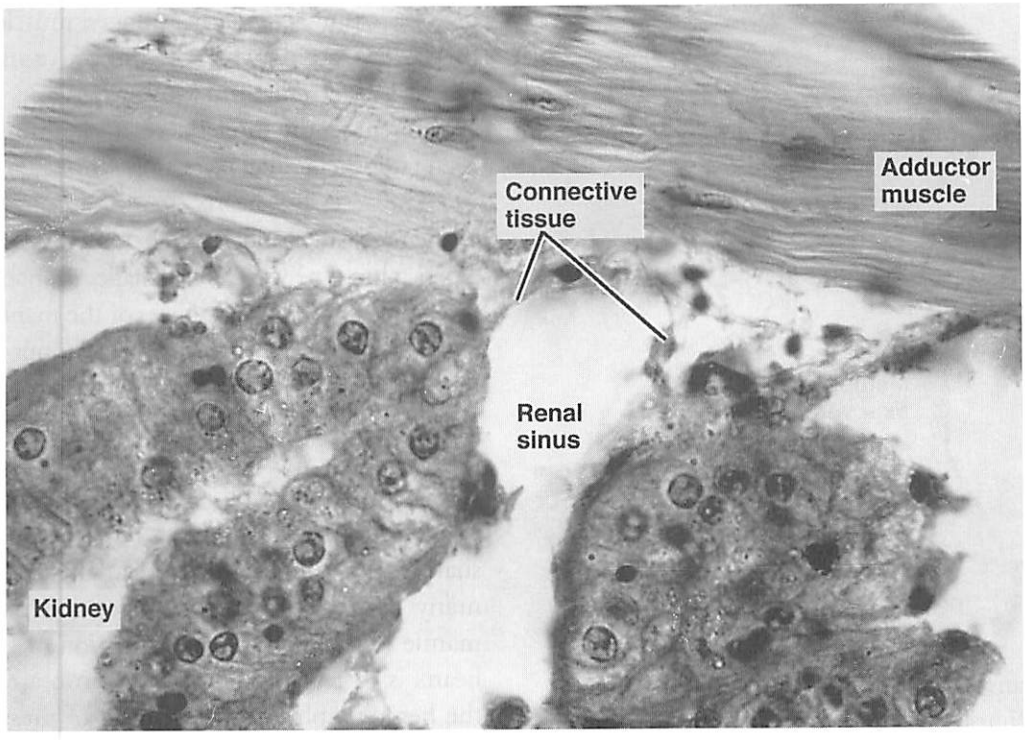


Figure 23. Transverse section through the adductor muscle and kidney. HFW = 110 µm. Note that kidney tubules are suspended from muscle fibers by delicate strands of connective tissue and are bathed directly by the renal sinus.

the hemolymph directly to the gills via the medial gill axis vein (Figs. 4B, 24). Anteriorly, the renal sinus drains into the branchial vein. Posteriorly, the renal sinus drains into the posterior pallio-branchial vein as well as the accessory hearts, organs that pump hemolymph to the mantle via the posterior circumpallial arteries (Fig. 25).

Accessory Hearts

Accessory hearts are paired Y-shaped structures fused to the mantle in the region of the cloacal chamber into which they project (Fig. 25). The larger branch of the "Y" runs from the posteroventral corner of the adductor muscle parallel to the gill axis to the point of fusion of the posterior left and right mantle lobes (Fig. 25); the smaller branch closely follows the course of the posterior aspect of the adductor muscle (Fig. 25). This smaller branch runs dorsally for about half the length of the adductor muscle then ends abruptly by entering the tissues of the mantle where it sends numerous branches to the shell-side of this organ (Fig. 25).

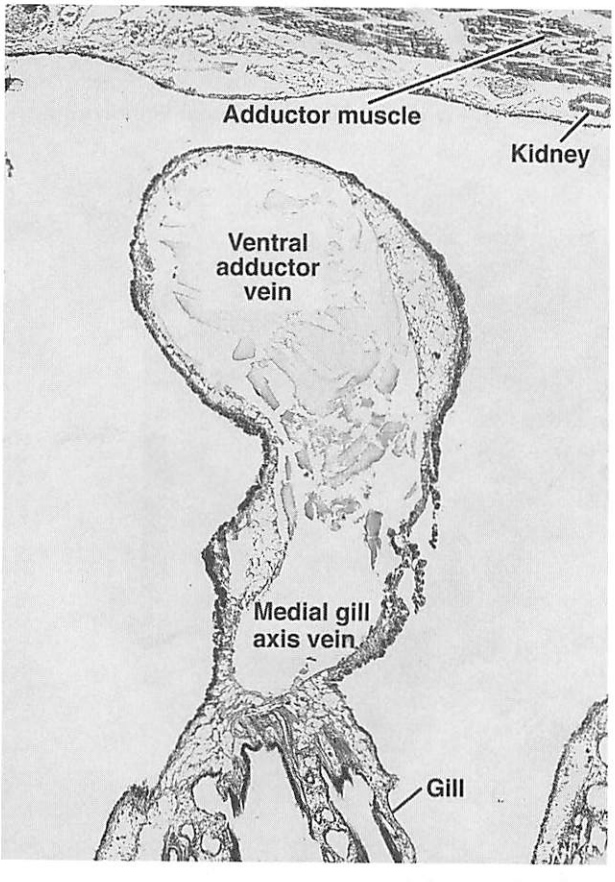


Figure 24. Transverse section (HFW = 1.5 mm) through the adductor muscle at the level of the ventral adductor vein; this vessel drains the adductor muscle and a portion of the kidney and empties into the medial gill axis vein.

My research has indicated that both major branches that constitute the accessory hearts are muscular tubes covered on their nonattached surface by a much-folded, low columnar ciliated epithelium that contains two types of unicellular glands: (1) a granular cell rich in neutral glycoproteins that stain strongly with the periodic acid Schiff stain and (2) a goblet-like cell that is strongly reactive for acidic glycocoproteins (= mucopolysaccharides). Just underneath the basement membrane of this epithelium is a layer of circular muscle, the subepithelial muscle, that follows every undulation of the epithelium (Fig. 26). Just central to the subepithelial muscle lies a thin layer of longitudinal muscles followed by a prominent layer of crisscrossing, circularly arranged muscle fibers (Fig. 26). A delicate network of connective tissue consisting of a fine reticulum woven between Leydig cells are present in the interstices between muscle fibers as is an endothelial-like layer (Fig. 26).

The accessory hearts drain the posterior portions of the renal sinus and pump hemolymph posteriorly in a peristaltic-type wave. The smaller branch delivers hemolymph to the pallial sinuses of the mantle lining the cloacal chamber; the larger branch pumps hemolymph into the mantle via the posterior circumpallial arteries (Fig. 25). The left and right accessory hearts communicate with one another via the circumpallial arteries (Fig. 25). Hemolymph is pumped, then, to the posterior circumpallial arteries that immediately bend sharply and run in an anterior direction giving off many branches to the tentacles and tissues of the mantle (Fig. 25). Thus, the function of the accessory hearts is to receive hemolymph from a region where the hemolymph is at a low head of pressure (having passed through the adductor and renal sinus systems) and send it to the mantle under pressure for oxygenation and return to the heart.

Left and right accessory hearts do not beat synchronously but seem to alternate regularly with one another; the average number of beats is 10 min^{-1} at 20°C, although the left accessory heart was observed to beat faster than the right (Hopkins 1934a). A bundle of pallial radial muscle, consisting of large coarse fascicles of longitudinally oriented fibers enclosing or partially surrounding a large radial nerve, projects into the lumen of the accessory hearts (Fig. 27). Similar bundles of radial muscles are found projecting into the mantle cavity in this area, each surrounded by a hemolymph vessel and enclosing a branch of the radial nerve (Fig. 28). Many authors (Hopkins 1936b; Elsey 1935; Nelson 1938) have pointed to the close similarity between the radial vessels of the mantle and the accessory hearts. In young spat (1 to 2 mm), all radial vessels of the mantle pulsate rhythmically but lose this ability with time. Thus, it appears that the accessory hearts are homologous to the pallial radial hemolymph vessels, most of which lose their ability to pulsate as the eastern oyster matures.

The Pallial Circulation

The mantle receives arterial hemolymph from the circumpallial arteries (Figs. 4, 25), the anterior pallial arteries, the posterior pallial arteries, the accessory hearts (Fig. 25), the median visceral artery, and the right visceral artery (Fig. 4A).

Pallial venous hemolymph is collected from the anterior half of the oyster by a complex series of branching vessels that empty into the branchial and pallio-cloacal veins (Figs. 16, 25); the pallio-cloacal

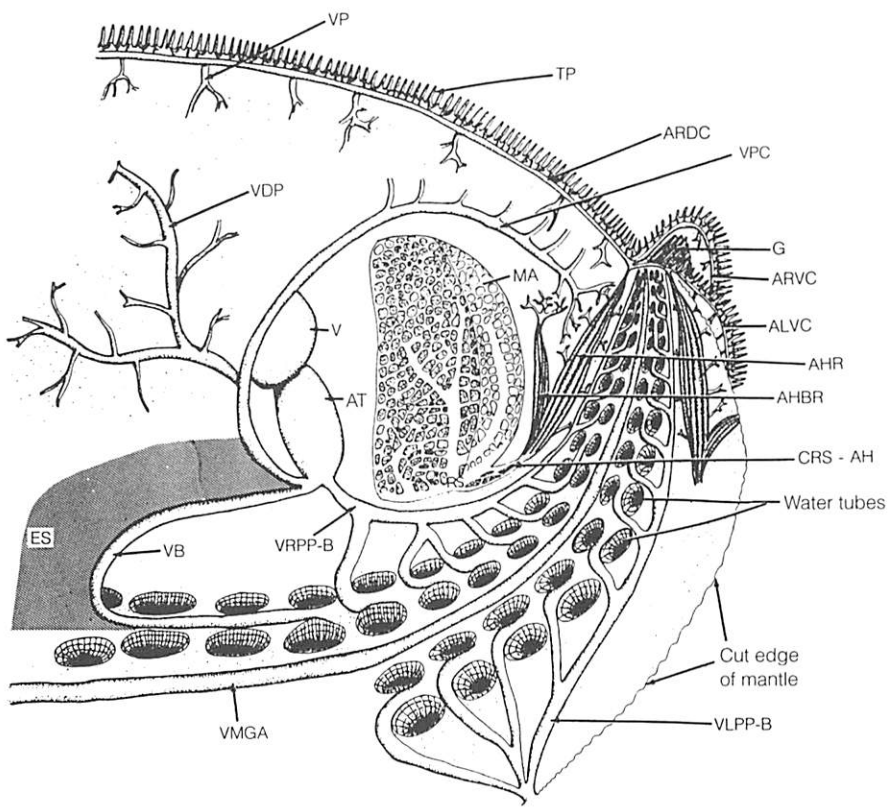


Figure 25. Composite drawing of hemolymph circulation in the posterior half of the oyster (× 5). Ventrally, the left mantle has been dissected away from its connection to the adductor muscle and stretched away from the body exposing water tubes and accessory hearts. Dorsally, the left mantle above the adductor muscle has been removed so the left dorsal circumpallial artery is not represented in figure. Viewed from left side. See appendix for list of abbreviated terms.

vein receives an especially heavy drainage from the anterodorsal portion of the mantle before returning hemolymph to the heart (Fig. 25).

In the posterior half of the oyster, the pallial drainage is divided into dorsal and ventral portions; the latter is drained by many elongate veins that empty into the posterior pallio-branchial vein whereas the dorsal pallial region is drained by the pallio-cloacal vein (Figs. 16 and 25). Interestingly, several of the larger veins, e.g., the pallio-cloacal vein and the pallial vessels that drain into it as well as the medial gill axis vein, have prominent muscular coats and exhibit contractions of a slow peristaltic type.

The mantle has an extensive system of hemolymph sinuses that ramify throughout this large organ, with sinuses occupying more volume than Leydig tissue in many regions. Thus, the mantle functions efficiently as an organ for gas exchange with the environment. The extensive subepithelial sinuses bring essential metabolites to both pallial epithelia to support shell building and repair on the one surface and ciliary activity and mucus production on the other.

Venous Return to the Heart

Hemolymph is returned to the systemic heart of the eastern oyster via three major veins. The pallio-cloacal vein drains the area of the cloacal chamber as well as large portions of the dorsal mantle (Figs. 16, 25). The posterior pallio-branchial vein drains the entire ventral face of the mantle in the posterior half

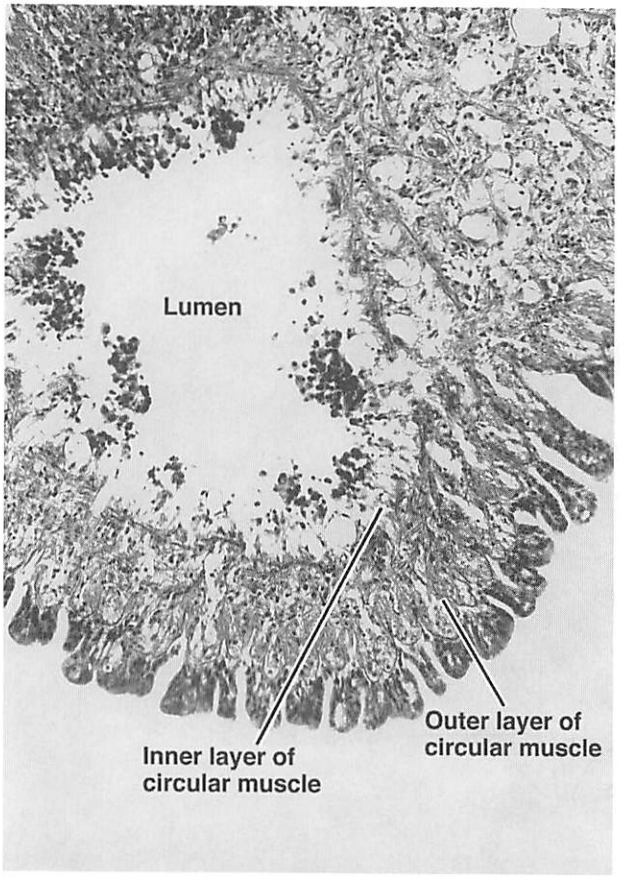


Figure 26. Transverse histological section of an accessory heart. Note prominent layers of circular muscle with fibers arranged in a criss-cross pattern. HFW = 287 µm.

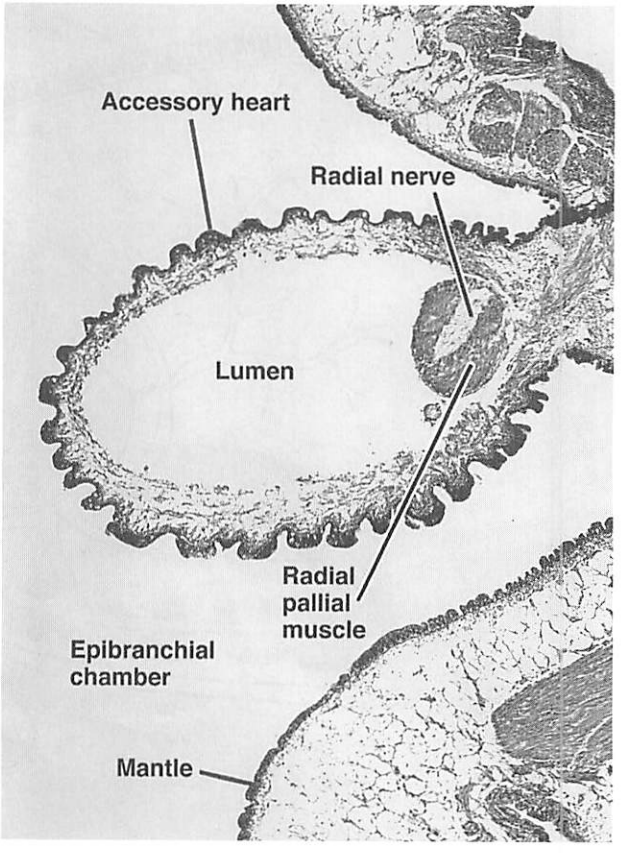


Figure 27. Transverse histological section of an accessory heart; note bundle of radial pallial muscle containing a branch of the radial nerve projecting into the lumen. HFW = 287 µm.

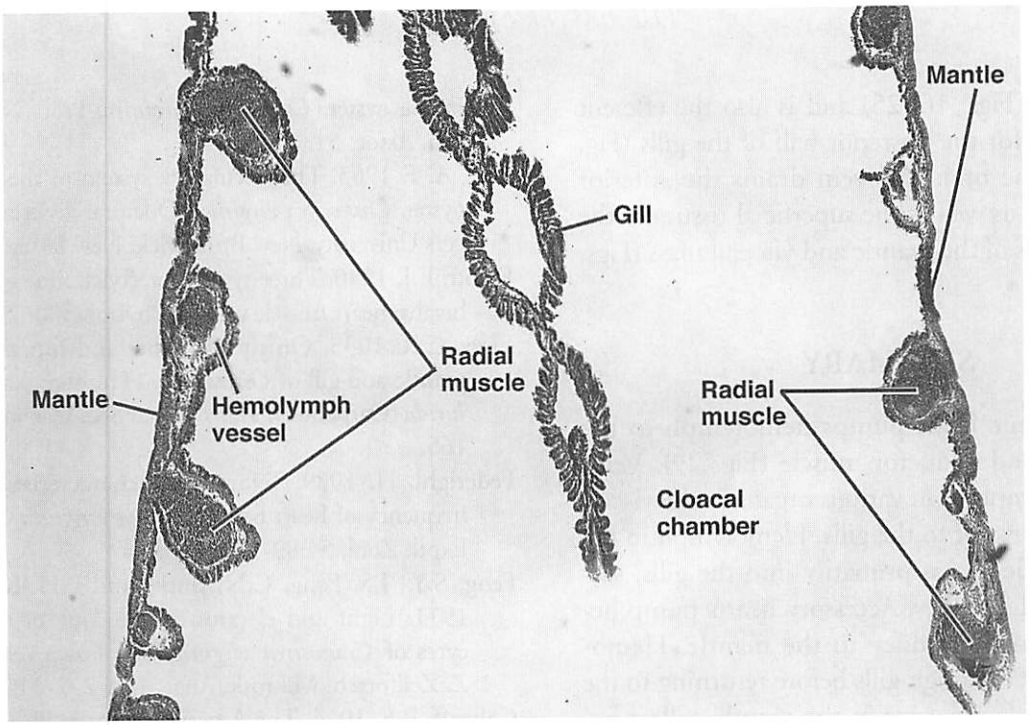


Figure 28. Transverse section of the cloacal chamber to show radial muscles of mantle, each with an associated hemolymph vessel. HFW = 1.6 mm.

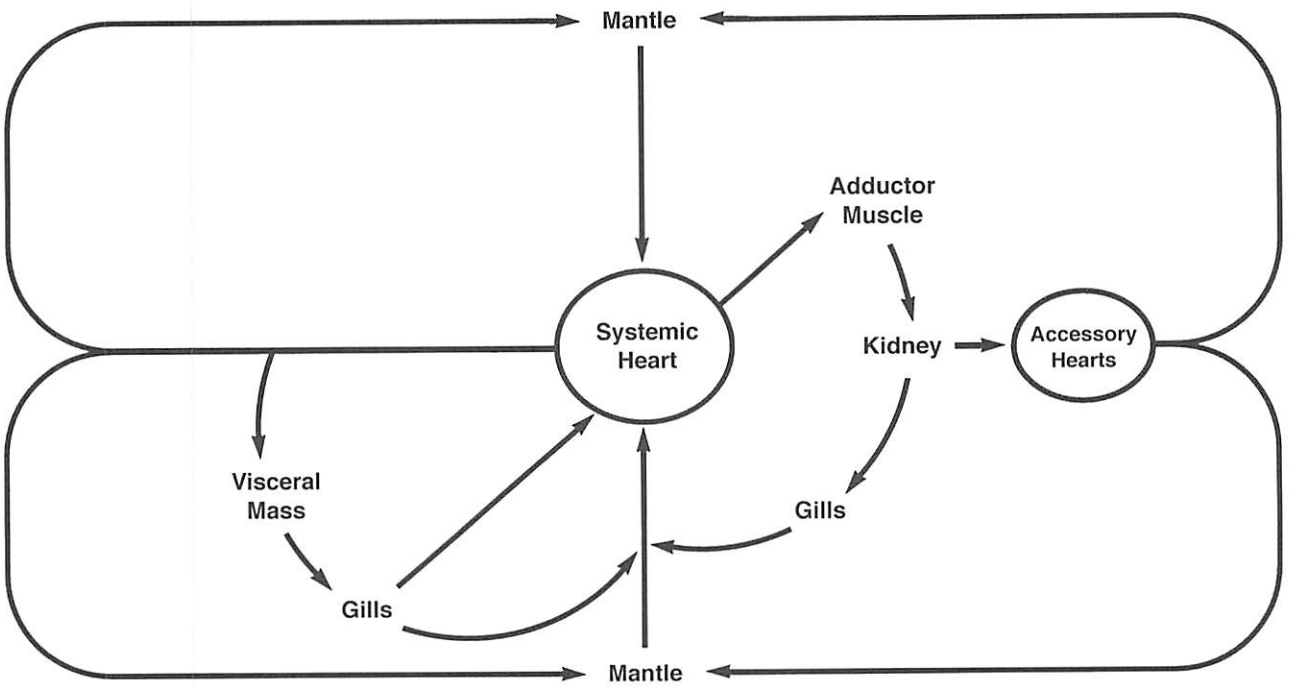


Figure 29. Schematic interpretation of the circulatory system of the eastern oyster. Note that all of the hemolymph supply of the visceral mass as well as most of the adductor muscle drains into the medial gill axis vein and then into the gills before returning to the heart. The mantle and the gills are the two chief organs for oxygen exchange with the environment. Both accessory hearts receive hemolymph from the adductor muscle and the renal sinus. Accessory hearts then pump hemolymph into the mantle after which it is returned to the heart.

of the animal (Figs. 16, 25) and is also the efferent branchial vein for the posterior half of the gills (Fig. 14). Finally, the branchial vein drains the anterior half of the gills as well as the superficial tissues of the anterior regions of the mantle and visceral mass (Figs. 14, 25).

SUMMARY

The systemic heart pumps hemolymph to the visceral mass and adductor muscle (Fig. 29). Veins collect hemolymph from various organs in the visceral mass and deliver it to the gills. Hemolymph in the adductor muscle drains primarily into the gills, secondarily into the kidney. Accessory hearts pump hemolymph from the kidney to the mantle. Hemolymph circulates through gills before returning to the systemic heart. Large veins in the mantle collect hemolymph and return it to the systemic heart (Fig. 29).

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APPENDIX

Abbreviations Used in Figures

AA – Anterior aorta
AALV – Anterior left visceral artery
AB – Anteroaortic bulb
ACDC – Common dorsal circumpallial artery
ACL – Common labial artery
ADA – Dorsal anterior arteries
ADP – Dorsal pallial arteries
AH – Hepatic arteries
AHBR – Branch of accessory heart
AHR – Right accessory heart
ALDC – Left dorsal circumpallial artery
ALLL – Left lateral labial artery
ALML – Left medial labial artery
ALVC – Left ventral circumpallial artery
AMV – Medial visceral artery
AOH – Oral hood artery
APA – Anterior pallial arteries
APG – Posterior gastric arteries
APLV – Posterior left visceral artery
APP – Posterior pallial artery
AR – Rectal artery
ARDC – Right dorsal circumpallial artery
ARLL – Right lateral labial artery
ARML – Right medial labial artery
ARV – Right visceral artery
ARVC – Right ventral circumpallial artery
AT – Atrium
AVV – Ventral visceral artery
CRS-AH – Connection of renal hemolymph sinus to accessory heart
ES – Epibranchial septum
HA – Accessory heart
G – Gill
I – Intestine
JAV – Atrio-ventricular junction
K – Kidney
KB – Bladder of kidney
M – Mantle
MA – Adductor muscle
P – Pericardial coelom
PA – Posterior aorta
PI-S – Peri-intestinal sinus
PLL – Left lateral palp
PP – Pyloric process (posterior process of the visceral mass)
RS – Renal sinus
SC – Slender cell
TP – Pallial tentacles
V – Ventricle
VAA – Anterior adductor veins
VALM – Anterior left medial visceral vein
VALV – Anterior left visceral vein
VARM – Anterior right medial visceral vein
VAP-B – Anterior pallio-branchial vein
VARV – Anterior right visceral vein
VB – Branchial vein
VDA – Dorsal adductor vein
VDP – Dorsal pallial vein
VLGA – Left lateral gill axis vein
VLLL – Left lateral labial vein
VLML – Left medial labial vein
VLPP-B – Left posterior pallio-branchial vein
VLV-A – Left viscero-adductor vein
VMGA – Medial gill axis vein
VMRV – Medial recto visceral vein
VP – Pallial vein
VPC – Pallio-cloacal vein
VPG – Posterior gastric veins
VPP-B – Posterior pallio-branchial vein
VR – Rectal veins
VRLL – Right lateral labial veins
VRML – Right medial labial vein
VRPP-B – Right posterior pallio-branchial vein
VRPV – Right posterior visceral vein
VR-V – Recto-visceral vein
VRV-A – Right viscero-adductor vein
VVA – Ventral adductor vein
WT – Water tube

Chapter 8

Hemocytes: Forms and Functions

THOMAS C. CHENG

INTRODUCTION

The circulatory system in molluscs, except for the cephalopods, is of the open type, that is, both the serum and hemocytes are not confined to the interior of the heart and vessels. These constituents of hemolymph also occur in sinuses and tissues. Furthermore, as a result of the periodic contraction of the muscular cardiac wall and the tissues surrounding the various sinuses, whole hemolymph is expelled from the body by diapedesis, the passage across membranous body surfaces (Cheng 1981). As discussed in later sections of this chapter, molluscan hemocytes are involved in many functions, including digestion and nutrient transport (Yonge 1923, 1926; Canegallo 1924; Takatsuki 1934a; Yonge and Nicholas 1940; Zacks and Welsh 1953; Wagge 1955; Owen 1966; Cheng and Cali 1974; Cheng and Rudo 1976a; Cheng 1977a; Feng et al. 1977), wound healing (Pauley and Sparks 1965; des Voigne and Sparks 1968; Pauley and Heaton 1969; Ruddell 1969; Sparks 1972, 1985), shell repair (Wagge 1951, 1955), excretion (Durham 1891; Orton 1923; Canegallo 1924; Martin 1983), and internal defense (Hardy et al. 1977a; Cheng 1981; Bayne 1983; Ratcliffe et al. 1985; Fisher 1986; Feng 1988). In view of these essential physiological functions, it is not surprising that considerable effort has been devoted to understanding the composition and exact functions of molluscan hemolymph. Because of the increased interest in shellfish diseases, especially those of oysters, during the past 25 years, the immunological roles of molluscan hemolymph have been investigated intensely, with considerable information pertaining to this aspect of hemolymph function now available.

The objective of this chapter is to present a synopsis of what is known about the morphology and functions of molluscan hemocytes, especially those of the eastern oyster, *Crassostrea virginica*. Throughout the text and tables, mean values are reported ± 1 SD, unless otherwise indicated.

Formed Hemolymph Elements

Interest in the identification and classification of bivalve hemocytes originated with Knoll (1893). He reported the occurrence of one type of hemocyte, designated as amoebocyte, in *Pectunclus* sp., *Solen* sp., *Unio* sp., and *Anodonta* sp. De Bruyne (1895) reported two categories of hemocytes, namely, granulocytes and lymphocytes, in *Mytilus edulis*, *Ostrea edulis*, *Unio pictorum*, and *Anodonta cygnea*.

Takatsuki (1934a), in a landmark study, investigated the cells of *O. edulis*. He reported the occurrences of two categories: granular leucocytes and lymphocytes (Table 1). Subsequent investigators have studied the hemocytes of several species of oysters: *O. edulis*, *Ostrea circumpicta*, *Crassostrea gigas*, and *C. virginica*, but there has been little agreement on the types of hemocytes that occur in oysters. Among the most detailed studies is that by Tanaka et al. (1961) who investigated *C. gigas*. These investigators, taking into consideration the shapes and sizes of cells and nuclei, position and staining intensity of nuclei, distribution of chromatin, presence or absence of nucleoli, nature of the

Table 1. Reported designations and characteristics of hemocytes of *Crassostrea virginica.*

Hemocyte types or designations	Dimensions (μm)	Diagnostic characteristics	Authority
Amoebocyte, leucocyte, or phagocyte			Stauber 1950
Granular cell or amoebocyte	6 (unspread)	Cytoplasmic granules neutrophilic or basophilic; bristle-like pseudopodia	Galtsoff 1964
Hyalinocyte	5 to 15 (diameter)	Cytoplasm with few granules; slow moving; pseudopodia lobose; basophilic	Galtsoff 1964
Granular leucocyte	10 to 20 (diameter)	Nucleus round to oval; cytoplasm with light or dense granules	Feng et al. 1971
Agranular leucocyte	8 (diameter)		
Type I		Large oval nucleus; scanty cytoplasm; little glycogen	Feng et al. 1971
Type II		Oval nucleus; moderate amount of cytoplasm; with clusters of glycogen granules	Feng et al. 1971
Type III		Spherical nucleus with dense chromatin	Feng et al. 1971
Granulocyte	10 ± 1 × 12 ± 1.2 (fresh)	With acidophilic, basophilic, refractile granules, or combinations of these; spike-like filopodia	Foley and Cheng 1972
Fibrocyte			
Primary	9.3 ± 1.6 × 8.2 ± 1.5 (spread)	With lobate nucleus and cytoplasmic vacuoles; few or no granules	Foley and Cheng 1972
Secondary	9.6 ± 1.4 × 9.4 ± 1.2 (spread)	With spherical or ovoid nucleus with few or no cytoplasmic granules but with vacuoles	Foley and Cheng 1972
Hyalinocyte	9.3 × 8.2 (spread)	Scanty cytoplasm with few or no granules; pseudopodia lobose	Foley and Cheng 1972
Granulocyte	Same as Foley and Cheng 1972	Same as Foley and Cheng 1972 except that fibrocytes considered as terminal granulocytes	Cheng 1975
Hyalinocyte	Same as Foley and Cheng 1972	Same as Foley and Cheng 1972	Cheng 1975
Granulocyte			
Large (~10%)	13 to 20 (diameter)		Renwrantz et al. 1979
Medium (40%)	9 to 13 (diameter)	Same as granulocyte of Foley and Cheng 1972	Renwrantz et al. 1979
Small (30%)	3 to 9 (diameter)		Renwrantz et al. 1979
Hyalinocyte		Same as hyalinocyte of Foley and Cheng 1972	Renwrantz et al. 1979
Type I	ca. 4 (diameter)	Agranular or slightly granular; with central rounded nucleus (ca.1.75 μm diameter);	Hawkins and Howse 1982

Table 1. (Continued.)

Hemocyte types or designations	Dimensions (μm)	Diagnostic characteristics	Authority
		cytoplasm moderately dense containing scattered organelles; frequently with juxtanuclear bodies (osmiophilic granules surrounded by double membrane-bound bodies) (ca. 400 nm diameter); multivesicular bodies often near juxtanuclear bodies	
Type II	ca. 6.5 (diameter) (oval shape)	Centric to eccentric nucleus (ca. 2.5 μm diameter) with few pseudopodia; cytoplasm often clear but may contain numerous glycogen granules, small clear vesicles, scattered mitochondria, and rough endoplasmic reticulum (RER); frequently with juxtanuclear bodies (see Type I); RER cisternae, dense vesicles, and lysosome-like bodies common	Hawkins and Howse 1982
Type III	ca. 8.5 to 10 × 6 (oval shape)	Eccentric nucleus (some with two); some with lipid-like vesicles; with paracrystalline inclusions in dilated RER cisternae; perinuclear space and RER cisternae connected; with dense-cored granules (275 to 800 nm diameter); with phagocytosed cellular debris; with numerous thin pseudopodia	Hawkins and Howse 1982
Type IV	ca. 11 (diameter)	Centric to eccentric nucleus; few thin pseudopodia; numerous clear-centered cytoplasmic granules (450 to 990 nm diameter); with numerous glycogen granules; with small dense cytoplasmic granules	Hawkins and Howse 1982
Basophilic granulocyte	20 to 30 (10)[a] (diameter)	Most obvious type; largest of all hemocytes; oblong/pleomorphic	McCormick-Ray and Howard 1991
Small granule granulocyte	20 to 30 (diameter)	Round, not as pleomorphic; with granules that are smaller, fewer, and less intensely stained than those of basophilic granulocytes	McCormick-Ray and Howard 1991
Eosinophic granulocyte	10 to 12 (diameter)	With pink or dark granules in pink or neutral cytoplasm	McCormick-Ray and Howard 1991
Refractive granulocyte		With refractive granules and neutral cytoplasm; interpreted to be dead or moribund cells	McCormick-Ray and Howard 1991
Lymphoid agranulocyte	10 to 15 (7)[a] (diameter)	Small; with strongly basophilic nucleus and cytoplasm; round and with large centric nucleus	McCormick-Ray and Howard 1991
Nonlymphoid agranulocyte		With slight to copious basophilic cytoplasm; nucleus centric to slightly eccentric; low nuclear:cytoplasm ratio	McCormick-Ray and Howard 1991

[a] Range; number within parentheses is the smallest diameter recorded (such cells are extremely rare).

perinuclear zone, and nature of the cytoplasmic granules, distinguished 12 types of cells in six categories. However, Tanaka et al. (1961) concluded that all of the subcategories of cells fall under two broad types: agranulocytic and granulocytic.

Feng et al. (1971), in a light and electron microscope study of oyster hemocytes, classified the cells of *C. virginica* as agranular and granular leucocytes which, in the presently accepted terminology (Cheng 1981), should be designated as hyalinocytes and granulocytes. Feng et al. (1971) recognized three categories of hyalinocytes (types I, II, and III) and one type of granulocyte (Table 1). According to these investigators, type I hyalinocytes are lymphocyte-like and are characterized by a relatively large and oval-shaped nucleus and scanty cytoplasm. Type II hyalinocytes include an oval nucleus similar to that of type I cells but have considerably more cytoplasm. Type III hyalinocytes have a spherical nucleus containing dense chromatin.

Although Feng et al. (1971) recognized one type of granulocyte, they believed that there are three distinct types of cytoplasmic granules that, when stained with Giemsa's stain, appear refractile, dark blue, and pink, respectively. As a result of electron microscope studies, they proposed that the refractile granules be designated type A, the dark blue ones type B, and the pink granules type C. They also reported that granulocytes may include one or more types of granules.

Cheng and Cali (1974) and Cheng et al. (1974) also conducted electron microscope studies of *C. virginica* granulocytes. Unlike Feng et al. (1971), they interpreted the cytoplasmic granules ("vesicles") to be of only one type.

By examining living hemocytes with a phase contrast microscope and hemocytes subjected to several fixatives and stains, Foley and Cheng (1972) concluded that there are three categories of hemocytes: granulocytes, fibrocytes, and hyalinocytes, with granulocytes commonly including mixtures of acidophilic, basophilic, and refractile granules.

Foley and Cheng (1974) later reported that granulocytes, fibrocytes, and hyalinocytes also occur in the hard clam *Mercenaria mercenaria.* In addition, Cheng and Foley (1975) reported that fibrocytes of *M. mercenaria* often include large aggregates of glycogen granules, mostly as rosettes. Also, there are large, electron-lucent, membrane delimited vesicles in the cytoplasm commonly enclosing arrays of concentric lamellae (digestive lamellae) and partially degraded cellular debris. As a result of these findings, Cheng and Foley (1975) proposed that fibrocytes are actually granulocytes that have undergone degranulation and are engaged in intracellular digestion of foreign material. The basis for this interpretation is that granulocytes of bivalves (especially those of *C. virginica*) that have endocytosed bacteria form digestive lamellae in phagosomes around the bacteria. The latter eventually become enzymatically degraded (Cheng and Cali 1974). Intracellular digestion of bacteria results in the isolation of bacterial carbohydrate constituents, which are converted to glycogen and eventually deposited in large aggregates within granulocytes (Cheng and Cali 1974). This finding was employed as a basis for interpreting fibrocytes to be granulocytes at a later stage of the endocytosis-intracellular degradation continuum. Consequently, it now appears that there are two categories of hemocytes in *C. virginica* (see Cheng 1974, 1984a), namely, granulocytes (Fig. 1) and hyalinocytes (Fig. 2).

Hyalinocytes of *C. virginica* are either agranular or slightly granular. Although they are capable of forming pseudopodia, those produced are lobopodial rather than filopodial as in the case of granulocytes. The staining characteristics of oyster hyalinocytes, like those of granulocytes, vary with the fixative and stain employed (Foley and Cheng 1972).

Hawkins and Howse (1982) identified ultrastructurally four types of hemocytes or "hemocyte-like cells" in hemolymph from the heart of *C. virginica.* Types I, II, and III are agranular whereas type IV is granular (Table 1). Cells comprising types I, II, and III are increasingly larger (Table 1). In addition, there are differences in the organelles and other intracellular structures (Table 1). According to Hawkins and Howse (1982), type III cells are phagocytic. Upon analysis, there is little doubt that what Hawkins and Howse (1982) have designated as types I, II, and III represent three ontogenetic stages of oyster hyalinocytes. According to the staging system of Cheng (1982), types I and II may be considered prohyalinocytes. Foley and Cheng (1975), based on a quantitative assay, determined that mature hyalinocytes of

C. virginica are capable of phagocytic activity leading to endocytosis, although not as avidly as granulocytes. Phagocytosis is defined as a sequence of events, namely attraction between foreign body and phagocyte, surface attachment, and endocytosis. Traditionally, pinocytosis is defined as the intake of soluble molecules by cells; however, definition of the upper dimensional limits of "soluble" molecules has rendered this term somewhat obsolete.

Hawkins and Howse (1982) stated that type III hemocytes, which are characterized by paracrystalline inclusions in the rough endoplasmic reticulum (RER) and perinuclear space (Table 1), are usually closely associated with the myocardial trabeculae and possibly are noncirculating. They hypothesized that, for this reason, type III cells have not been reported previously. Based on the work of Foley and Cheng (1975), I maintain that their type III cells are mature hyalinocytes. The nature and function of the paracrystalline inclusions remain to be investigated.

Hawkins and Howse (1982) stated that their type IV cells are typical oyster granulocytes. It is surprising, however, that they reported that these cells are not phagocytic, because earlier studies (e.g., Feng et al. 1971; Cheng 1975; Foley and Cheng 1975) have all shown granulocytes to be the most actively phagocytic. Perhaps this discrepancy rests with the fact that Hawkins and Howse (1982) studied granulocytes situated in the heart rather than in the general circulation. Intracardial cells *in vivo* may be at a different stage of maturity or may behave differently.

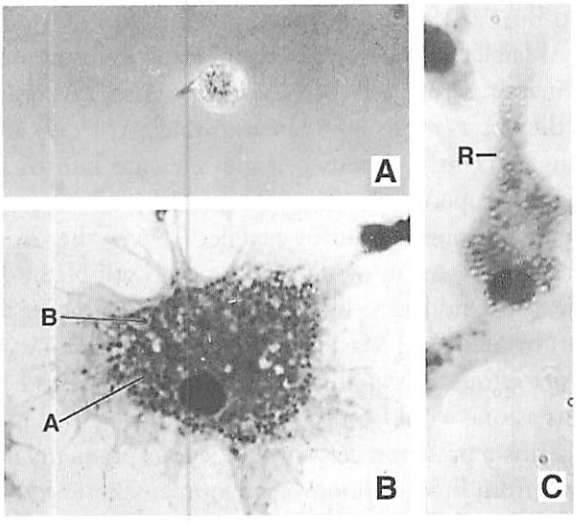

Figure 1. *Crassostrea virginica* granulocytes. (A) Granulocyte with single pseudopodium in fresh hemolymph. (B) Granulocyte fixed with 2.5% sea water glutaraldehyde, stained with Giema's stain, and photographed with a Kodak Wratton No. 58 green filter. [A] acidophilic granule, [B] basophilic granule. (C) Granulocyte identically fixed and stained but photographed without a filter. [R] refractile granule. After Cheng et al. (1974).

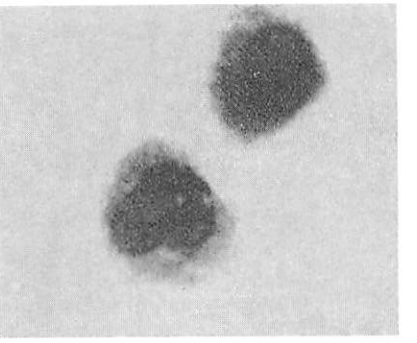

Figure 2. *Crassostrea virginica* hyalinocytes (methanol fixed, Giema stain; × 90). After Foley and Cheng (1972).

There is an additional type of cell in oysters that generally is not considered to be a true hemocyte. This type comprises the so-called serous or brown cells. Serous cells (Fig. 3) are produced in the Keber's glands which are situated on the auricles, or the mantle, or both, and which empty into the pericardial cavity. These glands are generally considered to be part of the excretory system (Martin 1983).

The pigment-bearing serous cells, once released from Keber's glands, occur not only in tissues but sometimes also within the closed portions of the circulatory system, and hence comprise a type of migrating and circulating cell. For this reason, some consider serous cells to be a type of hemocyte (for review, see Sindermann 1990). Their brownish-yellow coloration is due to pigmented cytoplasmic globules. Takatsuki (1934b) first reported that serous cells are amoeboid and capable of endocytosing carmine particles, and proposed that they are modified hemocytes.

The excretory function of serous cells was initially proposed by White (1942), based on Letellier's (1891) report of the occurrence of hippuric acid as

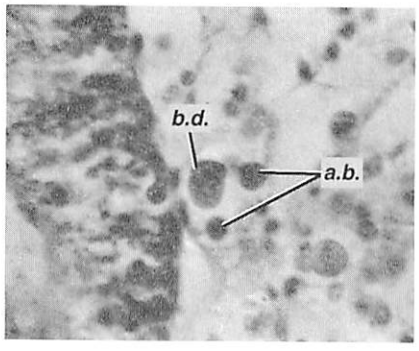

Figure 3. Section of *Crassostrea virginica* showing presence of acid mucopolysaccharide in certain serous cells [a.b.]. Notice dividing serous cell [b.d.], which does not include acid mucopolysaccharide. (Steedman's alcian blue stain, × 90). After Cheng and Burton (1966).

the principal component of the cytoplasmic globules associated with the pericardium of two bivalves, *Cardium* sp. and *Pecten* sp. White (1942) also noted that Takatsuki (1934b) had reported the occurrence of hippuric acid and other metabolic by-products in the pericardial fluid of *O. edulis*. As a result, White (1942) proposed that serous cells extract acids from the hemolymph, which they carry to the kidney (organ of Bojanus) via the ciliated renopericardial channels. In addition to this assumed function, White (1942) also proposed that serous cells serve as storage sites for fats and secreted shell-forming materials.

The function most frequently attributed to serous cells is the removal of degradation products of dead and moribund parasites and the metabolic byproducts of living parasites. Several investigators have noted increased pigmentation in *C. virginica* and other species of oysters infected with the annelid, *Polydora* spp., the oyster crab, *Pinnotheres ostreum*, and in *C. virginica* parasitized by the protozoan, *Haplosporidium nelsoni* (MSX). In each instance, the increased pigmentation reflects increased numbers of serous cells that presumably have taken up by-products of the parasites (see Cheng 1982, for review).

Mackin (1951) also noted an increase of serous cells in *C. virginica* parasitized by the protozoan *Perkinsus marinus*. He postulated that the increase is indicative of an imbalance in lipid metabolism, possibly due to an increase in the oxidative and reductive processes resulting from disease. Mackin's (1951) observations have been strengthened by the quantitative studies of Stein and Mackin (1955). Similarly, Cheng and Burton (1965, 1966) noted that, although serous cells are present in nonparasitized *C. virginica*, their number is increased in oysters parasitized by sporocysts of the trematode *Bucephalus* sp. More recently, Scro and Ford (1990) also reported an increase in the number of serous cells in *C. virginica* infected with *H. nelsoni*. Moreover, they reported a substantial amount of free ribosomes and RER in these cells and the occurrence of globules outside of serous cells.

Another function attributable to serous cells is the endocytosis of the degradation products of unreleased gametes in gonads, although this phenomenon is open to question. It may be that the cells involved are phagocytic hemocytes.

Serous cells found in tissues of *C. virginica* vary greatly in size. Each cell may measure from 4 to 18 μm in diameter. The cytoplasm is usually so packed with yellow-brown to dark brown globules that the nucleus becomes masked. These cells have been observed to undergo division in oyster tissue (Cheng and Burton 1966).

Haigler (1964) reported lipid and tyrosine-rich protein in serous cells of *C. virginica*. Also, as a result of the observations by Stein and Mackin (1955) and Haigler (1964), serous cell globules are known to possess properties similar to those of lipofuchsins although, as pointed out by Haigler (1964), the "lipofuchsin" present in oyster serous cells is soluble in dilute acids and bases. This is contrary to the earlier report by Stein and Mackin (1955) that the globules of oyster serous cells are insoluble in dilute acids and alkalis as one would expect of true lipofuchsins. Lipofuchsins represent a class of lipogenous pigments derived from lipid or lipoprotein sources (Pearse 1961) or fatty acids (Lillie 1954).

The complexity of the chemical composition of serous cell globules is further indicated by Cheng and Burton (1966) who reported that only some of the serous cells in *C. virginica* are periodic acid-Schiff (PAS)-positive and diastase-resistant. Thus, the chemical nature of this material could be a mucoprotein, a glycoprotein, a glycolipid, or a sphingolipid. Because Haigler (1964) has shown that serous cells are com-

posed of a complex of lipid and protein with properties similar to those of lipofuchsins, it would appear that the PAS-positive and diastase-resistant material can be classified as sphingomyelin. Also, the PAS-positive reaction is not due to the presence of a 1:2-glycol-containing carbohydrate but to the presence of a primary acylated amine adjacent to a hydroxyl group that is capable of reacting with periodic acid. In addition to sphingomyelins, Cheng and Burton (1966) also demonstrated acid mucopolysaccharides in serous cells of medium size (6 ± 2 μm).

Origin and Development of Hemocytes

Origin

The hemopoietic site(s) of oyster hemocytes remain(s) uncertain. What is known about the origin of hemocytes of bivalves has been summarized by Cheng (1981). Cuénot (1891) initially suggested that bivalve hemocytes may have their origin in special "glande lymphatiques" at the base of gills. Morton (1969) suggested that the circadian rhythm of adductor muscle activity and quiescence in the zebra mussel, *Dreissena polymorpha*, may be related to the formation of at least some of the hemocytes. Although no satisfactory account has yet been published relative to the hemopoietic site(s) in bivalves, including oysters, the generally accepted belief is that hemocytes arise from differentiation of connective tissue cells. Even this idea requires experimental testing.

Development

Some investigators believe that the different types of at least some cells may represent ontogenetic stages of a single general type (see Cheng 1981 for review). Although I agree that there are different types of hemocytes, I am also in agreement with Feng et al. (1971) that it is unknown if different stages occur among circulating hemocytes. Mix (1976), Moore and Lowe (1977), and Cheng (1981) have provided detailed accounts of the ontogeny of bivalve hemocytes. In addition, Cheney (1969), in an unpublished dissertation, has presented some information, and Balouet and Poder (1979) and Auffret (1988, 1989) have presented additional ontogenetic schemes for bivalve hemocytes. All of these accounts are essentially based not on direct evidence but on interpretative evaluations. Because the publication by Moore and Lowe (1977) was based on the cells of the blue mussel, *M. edulis*, it will not be considered at any length herein. A summary has been provided by Cheng (1981).

Mix (1976), who based his interpretations on the data of several investigators (Galtsoff 1964; Tripp et al. 1966; Cheney 1969; Ruddell 1969, 1971a, b; Cheng and Rifkin 1970; Feng et al. 1971; Foley and Cheng 1972; Mix and Tomasovic 1973), proposed that there are four "compartments" during which "cell renewal" or, more appropriately, ontogenesis of bivalve hemocytes, primarily those of oysters, occurs (Fig. 4). Mix (1976) postulated the occurrence of a leucoblast, which represents the "stem cell compartment," and which differentiates into a hyalinocyte, which in turn represents the "proliferating compartment" (Fig. 4). Subsequently, these hyalinocytes may differentiate into granular hyalinocytes or one of two classes of intermediate cells. The granular hyalinocytes and the intermediate cells are what Mix (1976) has designated the "maturing compartment" (Fig. 4). Mix (1976) proposed that further differentiation of the components of this compartment leads to the formation of basophilic granulocytes and acidophilic granulocytes, fibroblasts, and myoblasts from one of the two intermediate cells, and pigment cells from the second type of intermediate cells. The basophilic and acidophilic granulocytes, together with fibroblasts, myoblasts, and pigment cells, comprise what Mix (1976) has designated the "functional compartment" (Fig. 4). Finally, in what Mix considers the "cell loss compartment," all of the cells of the functional compartment contribute to defense reactions, with some loss through senescence and diapedesis (Fig. 4).

Mix's (1976) interpretations are not without merit because a part of the experimental aspect of his report showed that stem cell leucoblasts divide as do hyalinocytes of either the proliferating or the maturing compartment. Nevertheless, there is evidence that suggests that Mix's (1976) interpretations may not be correct. For example, some eastern oyster granulocytes include

both basophilic and acidophilic granules (Foley and Cheng 1972) and hence could be interpreted to represent an intermediate stage between basophilic and acidophilic granulocytes. Also, what had been designated as molluscan fibrocytes are now believed to be granulocytes that have undergone degranulation and are performing intracellular digestion (Cheng and Foley 1975).

Cheng (1981, 1983a) presented another interpretation of the ontogeny of bivalve hemocytes (Fig. 5). He postulated that a hypothetical granuloblast produces young granulocytes, or progranulocytes. A progranulocyte is characterized by (1) being the smallest of the circulatory cells of the granulocyte line, (2) having relatively few cytoplasmic granules, (3) being not actively phagocytic but capable of phagocytosis, (4) possessing a basophilic nucleus and cytoplasmic granules, and (5) producing few pseudopodia when spread. The progranulocyte differentiates into a granulocyte I (Fig. 5). A granulocyte I is characterized by (1) being of medium size, (2) having the presence of numerous basophilic, acidophilic, or refractile granules, or mixtures of two or more of these, (3) being actively phagocytic, (4) producing filopodial pseudopodia, (5) being capable of contributing to clumping, (6) having the inclusion of increased numbers of such organelles as Golgi, rough endoplasmic reticulum, lipid droplets, and lysosomes, and (7) having relatively high levels of acid hydrolase activity.

According to this ontogenetic scheme (Cheng 1981, 1983a), each granulocyte I matures into a granulocyte II (Fig. 5). The latter is characterized by (1) being the largest of the granulocyte line, (2) including large numbers of cytoplasmic granules, mostly, if not all, acidophilic, (3) including some cytoplasmic vacuoles, which are residues of the degranulation process (Foley and Cheng 1977), (4) being very actively phagocytic, (5) producing large numbers of semi-permanent filopodia when spread, (6) having the presence of Golgi, smooth and rough endoplasmic reticula, lipid bodies, and lysosomes (the granules of light microscopy), (7) being actively involved in clumping, and (8) having the highest levels of acid hydrolase activity.

If a granulocyte II has endocytosed foreign materials and intracellular degradation has occurred, it becomes a spent granulocyte (Fig. 5). Such a cell is characterized by (1) few filopodia, commonly at two poles, (2) few cytoplasmic granules, (3) large numbers of cytoplasmic vacuoles of various sizes and shapes, (4) lower levels of lysosomal hydrolase activities, and (5) presence of phagosomes commonly including digestive lamellae and amorphous, partially degraded granules in the cytoplasm. Under certain pathologic

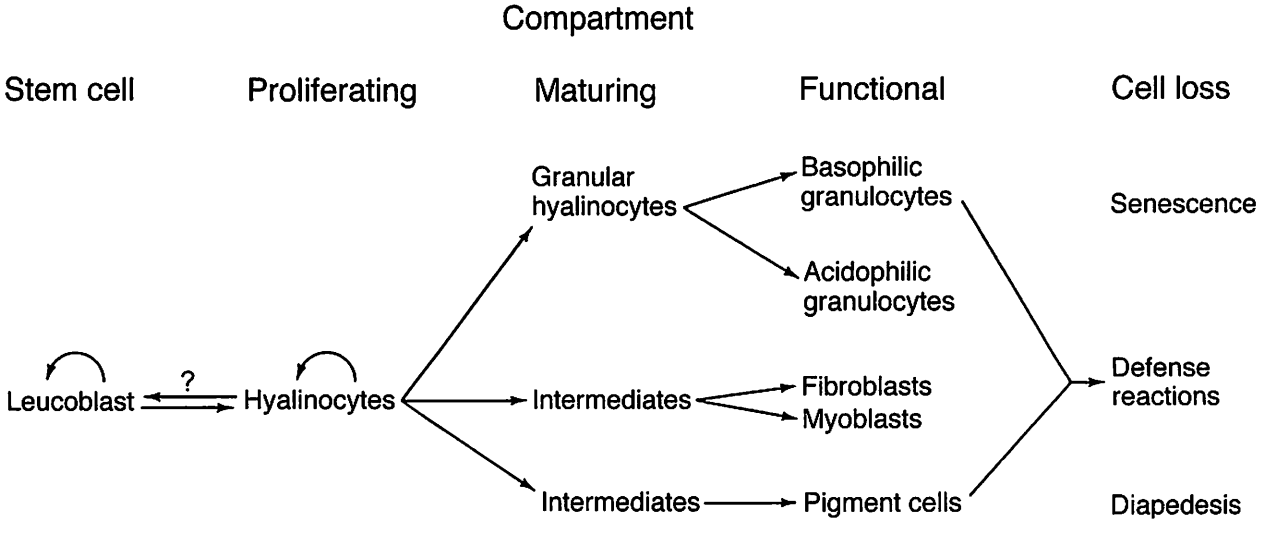

Figure 4. Mix's (1976) generalized model of hemocyte ontogenesis in bivalves. Arrows indicate the direction of development from one compartment to another.

conditions, such as during post-mortem change or rejection of incompatible grafts, several type II granulocytes in oysters (Sparks and Pauley 1964) and other molluscs (Cheng and Galloway 1970; Jourdane and Cheng 1987) may become fused to become a multinuclear macrocyte (or giant cell) (Fig. 5).

According to Cheng (1981, 1983a), a second ontogenetic series is represented by the hyalinocyte line (Fig. 5). Just as a granuloblast has been postulated for the granulocyte line, so has a hypothetical hyalinoblast been proposed for the hyalinocyte line. Cheng (1981, 1983a) has postulated that the hyalinoblast is capable of dividing and differentiating into young hyalinocytes, designated prohyalinocytes. Such a cell is characterized by (1) a relatively large nucleus embedded in a small volume of cytoplasm (i.e., a high nuclear-cytoplasmic ratio), (2) few cytoplasmic granules, (3) few or no lobopodia, and (4) being essentially basophilic. The prohyalinocyte matures into a hyalinocyte (Fig. 5), which has (1) a relatively small volume of cytoplasm surrounding a large nucleus (i.e., a high nuclear-cytoplasmic ratio), (2) few cytoplasmic granules, and (3) the characteristic of moving by producing a small number of lobopodia.

Finally, Cheng (1981, 1983a) has proposed that serous cells comprise a third ontogenetic line (Fig. 5). As stated, these cells are formed in Keber's glands and the youngest ones (i.e., those still within the gland or very recently expelled) are characterized by (1) being essentially nonmotile, and (2) having the presence of large, light to dark-brown cytoplasmic pigment globules. Aspects of the chemical nature of these globules have been discussed earlier. The youngest serous cells are capable of dividing and developing into medium serous cells. The latter include more pigment globules as well as acid mucopolysaccharide (Cheng and Burton 1966). Young and large serous cells do not include acid mucopolysaccharide. Large serous cells are tightly packed with numerous pigment globules (Fig. 5).

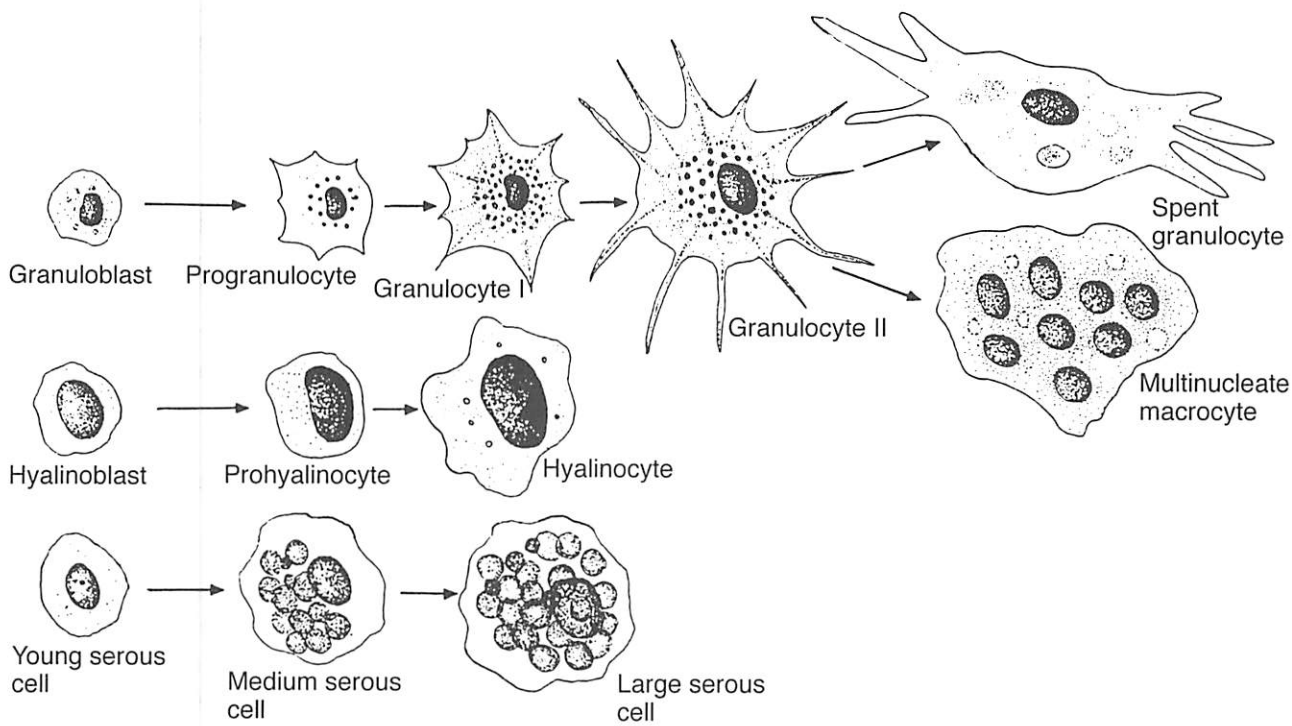

Figure 5. Diagram of hypothetical ontogenetic lineage of bivalve hemocytes. See text for description of each type. After Cheng (1981).

Balouet and Poder (1979) hypothesized that both hyalinocytes and granulocytes arise from the same stem cells (also designated as "intermediate cells"). According to Auffret's (1988, 1989) scheme, hyalinocytes arise from prohyalinocytes, in agreement with Cheng's (1981, 1983a) scheme. The difference lies in Auffret's (1988) idea that an "agranular prohemocyte" matures into an "agranular hemocyte" that is different from a hyalinocyte. His agranular hemocyte is postulated to mature into a "granular hemocyte" in *O. edulis* but into a "basophilic granular hemocyte" or an "acidophilic granular hemocyte" in *C. gigas*. This scheme is difficult to accept because there is no reason to believe that *O. edulis* and *C. gigas* have different hematologic developmental systems. Also, as stated, both acidophilic and basophilic granulocytes are known to occur in the same oyster and some cells include both types of granules. Because oyster hemocytes, especially granulocytes, are known to perform intracellular digestion, it should be recalled that in amoebae and ciliates there is a basophilic phase that follows an acidophilic phase in food vacuoles during intracellular digestion. A comparable situation may exist for molluscan granulocytes.

Subcategories of Granulocytes

There is evidence that subcategories of granulocytes exist. Specifically, Renwrantz et al. (1979) reported that granulocytes of *C. virginica* can be divided into three distinct morphometric subpopulations: small granulocytes (3 to 9 μm diameter), medium granulocytes (9 to 13 μm), and large granulocytes (13 to 20 μm). On the other hand, Cheng et al. (1980), using density gradient centrifugation and lectin identification of surface receptors, recognized four subpopulations of *C. virginica* granulocytes: subpopulation 1, consisting of cells measuring 3 to 5 μm in diameter; subpopulations 3 and 4, consisting of cells measuring between 10 and 14 μm; and subpopulation 5, consisting of aggregates of granulocytes and a few large cells. Subpopulation 2 consists of hyalinocytes.

Cheng et al. (1980) suggested that subpopulation 1 cells are the small granulocytes of Renwrantz et al. (1979) and the progranulocytes of Cheng's (1981) ontogenetic scheme. They also suggested that subpopulation 3 cells are the medium granulocytes of Renwrantz et al. (1979) and the granulocytes I of Cheng's (1981) scheme, whereas subpopulation 4 cells are the granulocytes II of Cheng (1981). The nature of the cells corresponding to the large cells of subpopulations is uncertain.

By employing various lectins (concanavalin A [Con A], wheat germ agglutinin [WGA], phytohemagglutinin [PHA, M form], *Ricinus communis* seed extract, *Ulex europaens* extract, *Soya* sp. extract, *Helix pomatia* albumin gland extract, *Cepaea nemoralis* albumin gland extract, and *Axinella polypodes* extract), Cheng et al. (1980) ascertained that hemocytes belonging to subpopulations 1, 3, and 4 are agglutinated with Con A and extracts of the albumin glands of the gastropods *H. pomatia* and *C. nemoralis*. Hyalinocytes (subpopulation 2) are also agglutinated by the same three lectins as well as WGA. By applying the Con A-peroxidase cytochemical technique, Cheng et al. (1980) determined that about 20% of the granulocytes of subpopulations 1 and 3 do not possess Con A-binding sites and only 18% of the large cells comprising subpopulation 5 possess such sites. These results suggest that the subpopulations of *C. virginica* granulocytes (immature and mature), distinguishable by their dimensions (Renwrantz et al. 1979) and densities (Cheng et al. 1980), may be further subdivided by differences in specific surface binding sites. The latter implies functional differences between hemocyte subpopulations.

The lectin-binding sites (receptors) on the surfaces of *C. virginica* granulocytes are capable of capping, i.e., the reaction product is concentrated at one or both poles of the cell (Fig. 6) and patching, i.e., the reaction product is spaced as patches over the entire cell surface (Fig. 7). Furthermore, at least two categories of granulocytes are indicated by differences in the percentages of capping and patching cells at permissive (21°C) (i.e., ideal) and nonpermissive (4°C) (i.e., non-ideal) temperatures (Table 2) among other characteristics (Yoshino et al. 1979). The notion that Con A capping may be associated with cell motility is suggested by the consistent change in cell morphology from spherical (at 4°C) to irregularly oblong and elongate (at 21°C) and the concomitant redistribution of receptors from diffuse (4°C) to capped (21°C).

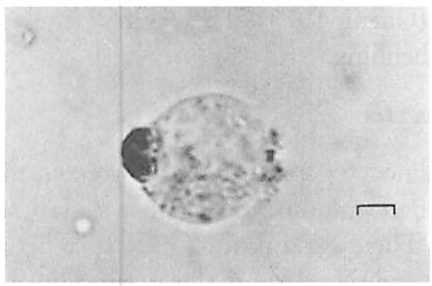

Figure 6. *Crassostrea virginica* granulocyte of the so-called large subpopulation with area of ligand capping associated with cell membrane protuberance. Bar = 2 μm. After Yoshino et al. (1979).

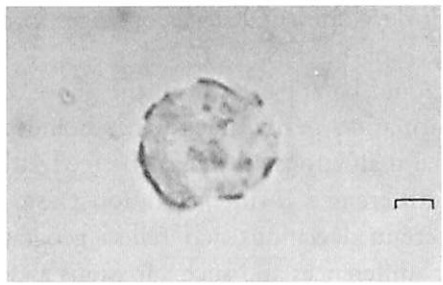

Figure 7. Patched *Crassostrea virginica* granulocyte of large subpopulation. Bar = 2 μm. Receptor distribution is indicated by the dark-staining reaction product, horseradish peroxidase. After Yoshino et al. (1979).

McCormick-Ray and Howard (1991), who employed Wright's stain on formalin-fixed cells, reported four types of granulocytes and two types of hyalinocytes (agranulocytes) in *C. virginica*. Among granulocytes, the first type, designated as basophilic granulocytes, is the largest and most obvious. The second type, designated small granule granulocytes, is similar in size and shape to basophilic granulocytes, but is not as pleomorphic and the cytoplasmic granules are smaller, less intensely stained, and usually fewer in number. The third type, known as eosinophilic granulocytes, contains pink to dark granules embedded in pink or neutral cytoplasm. In addition, McCormick-Ray and Howard (1991) reported a fourth type they designated refractive granulocytes, characterized by the presence of refractile granules and neutral cytoplasm. McCormick-Ray and Howard (1991) considered the refractive granulocytes, which are scarce, to be dead or moribund cells.

The two types of hyalinocytes (agranulocytes) described by McCormick-Ray and Howard (1991) are termed lymphoid and nonlymphoid, based on size differences and the nature of the cytoplasm. Lymphoid cells are small (7 to 15 μm), possess densely basophilic nuclei and cytoplasm, are generally round, and have a large nucleus that is usually centric. Nonlymphoid hyalinocytes are large (15 to 30 μm), with slight to copious basophilic cytoplasm. Their nuclei range from centric to slightly acentric. Some of these cells were reported to be oblong or round and to resemble small granulocytes, but without granules. Others are flattened and with either an irregular or a round, slightly ruffled, periphery. Both cell forms possess neutrophilic cytoplasm and a small nuclear:cytoplasm ratio. A few cytoplasmic granules are sometimes present in those cells with a ruffled periphery.

Because the classification scheme proposed by McCormick-Ray and Howard (1991), as with all earlier studies, is based on interpretive evaluation, it is not possible to state if one cell type is the progenitor of another. The four types of granulocytes and two types of hyalinocytes identified by these authors may

Table 2. Mean percentages (±1 SE) of large subpopulation (LS) and small population (SS) granulocytes of *Crassostrea virginica* producing Con A receptor caps or patches at permissive (21°C) and nonpermissive (4°C) capping temperatures. After Yoshino et al. (1979). N = number of replicates.

Incubation temperature (°C)	N	LS Granulocytes		SS Granulocytes	
		Capped (%)	Patched (%)	Capped (%)	Patched (%)
21	6	22.2 ± 1.3	14.6 ± 1.3	11.0 ± 0.6	14.7 ± 1.6
4	6	3.7 ± 1.0	2.4 ± 1.3	2.2 ± 0.3	1.3 ± 0.4

represent developmental stages or stages in the functional cycle of the cells.

It should be apparent from the above, especially the information pertaining to true hemocytes, i.e., cells other than serous cells, that observed variations as well as differences in interpretation exist. Furthermore, certain deviations may reflect geographic and temporal differences and such variations as age of the oysters, developmental stages of the hemocytes, and ambient factors. The definitive solution rests with the identification of the hematopoietic tissue(s), maintaining it in culture, and studying cell lineage *in vitro.*

Hemocyte Behavior

Foley (1974) reported that oyster hemocytes portray characteristic behavior *in vitro.* These behavioral patterns can be employed in distinguishing granulocytes from hyalinocytes. In brief, when first placed on a glass slide, both categories of hemocytes display tufts of filopod-like protrusions, each about one-third to one-half of the diameter of the main cell body in length. When examined with phase-contrast microscopy, granulocytes are highly refractile whereas hyalinocytes are only slightly refractile. The cells commonly form aggregates comprised of two to several hundred cells that may be of the same or different types.

Often, within 5 min after the aggregates are formed at 22°C, exomigration of cells (i.e., migration away from the clump) commences. Concurrently, the earliest hemocytes to undergo exomigration begin to adhere and flatten against the glass. The spreading behaviors of granulocytes and hyalinocytes are distinct and both are described below.

Granulocytes

In the case of granulocytes, as adherence and spreading occur, the cytoplasmic organelles, including granules, become confined to the endoplasm (Fig. 8). Adherence of the cell to glass is not due exclusively to gravity because cells also adhere to the underside of coverglasses. In addition to spreading, granulocytes produce fine, spike-like filopodia extending from the endoplasm past the usual periphery of the ectoplasm. These projections are semi-rigid, each supported by a fascicle of about 290 microtubules (Cheng 1975). They shift laterally without marked bending.

Hyalinocytes

Hyalinocytes spread more slowly and to a lesser extent than granulocytes against a glass substrate (Fig. 9). There is no distinct difference between endoplasm and ectoplasm in these cells. The pseudopodia produced are lobose or pointed, but do not include a supporting fascicle of microtubules.

Cytoplasmic Fragments

In addition to granulocytes and hyalinocytes, anucleate cytoplasmic fragments are commonly encountered. These have been observed to originate as tips of pseudopodia of spread cells that, for some undetermined reason, break off. These fragments are capable of producing fine, elongate filopodia along their periphery and may exhibit limited migration (Foley 1974). It is not known whether these fragments are capable of phagocytosis.

Cell Migration

Oyster hemocytes are mobile. They glide along a solid or semisolid substrate aided by pseudopodia. Granulocytes are considerably more mobile than hya-

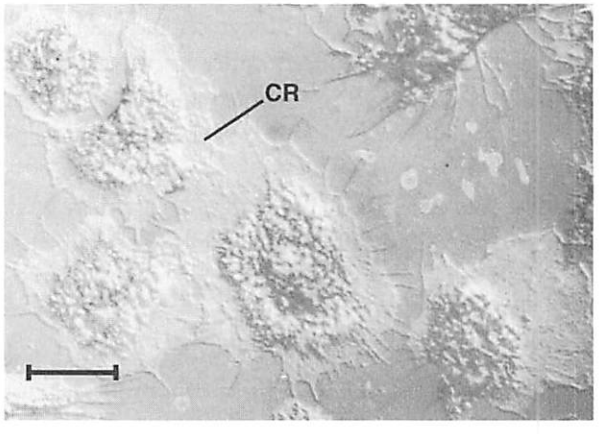

Figure 8. Spread *Crassostrea virginica* granulocytes showing cytoplasmic granules limited to endoplasm and presence of supporting fascicle of microtubules [CR]. 2.5% sea water-glutaraldehyde fixation, Giema's stain, Nomarski optics. Bar = 4.5 µm. After Foley and Cheng (1972).

linocytes. The mechanism(s) governing migration has not been studied.

The migration rates of oyster hemocytes may fluctuate throughout the year and the cells' ability to spread is influenced by the annual temperature cycle (Fisher et al. 1989). Bayne (1976) and McCormick-Ray (1987) have also reported that molluscan hemocytes are involved in other seasonal life cycle functions such as spawning, gametogenesis, growth, and buildup of energy reserves. The role of hemocytes in these functions is primarily in the regulation of nutrient flow.

Fisher and Newell (1986) reported that salinity affects granulocyte locomotion. Specifically, they found that locomotion of hemocytes of *C. virginica* acclimated to low salinities is retarded by acute increases in salinity and enhanced by decreased salinities. Continued retardation of low-salinity oyster hemocytes after a month or more of exposure to high salinity implies that hemocytes may carry an imprint of previous salinity exposure. Also, Fisher and Newell (1986) demonstrated that acute *in vitro* increases in salinity lengthens the time for hemocytes from oysters acclimated to low salinities to spread. This implies that an iso- or hyperosmotic cell is prerequisite for cell spreading or that volume regulation takes precedence over spreading in the case of cellular mechanisms or energy used for both activities.

Contrary to the earlier contribution by Fisher and Newell (1986), Fisher and Tamplin (1988) reported that during a year of high salinity, hemocytes from *C. virginica* collected in an estuary were retarded in their spreading at low salinities. They rationalized this difference by claiming that it is due to different ambient conditions. Nevertheless, Fisher (1988) stated (1) regardless of ambient or acclimation salinity, an acute increase in salinity results in a proportionally longer spreading time, and (2) an acute decrease in salinity does not affect spreading time except when the hemocytes are placed in very low, non-physiological salinities, i.e., < 10 ppt.

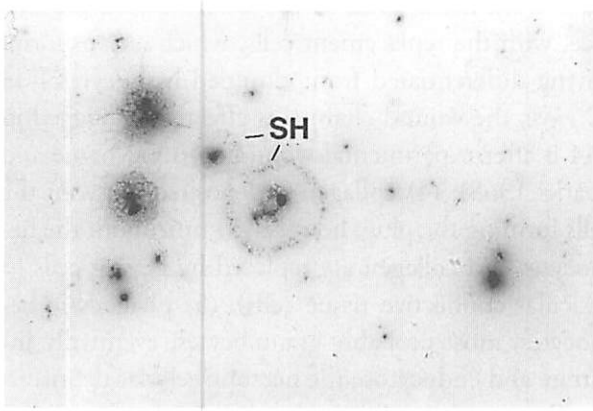

Figure 9. Two spread hyalinocytes [SH] of *Crassostrea virginica*. × 60. After McCormick-Ray and Howard (1991).

Fisher (1988) reported that temperature also influences the spreading of oyster hemocytes. He found that higher temperatures enhance spreading, i.e., spreading time is briefer; however, if the higher temperature creates stress, spreading time is increased. Thus, hemocytes from estuarine *C. virginica* have longer spreading times in the summer and early autumn than during lower-temperature spring conditions.

Because active phagocytosis, especially surface attachment and endocytosis, involves the migration and spreading of hemocytes, especially if the so-called Bang mechanism (see p. 317) is involved in endocytosis, it follows that both temperature and salinity can influence cell-mediated immunity.

Relative to the mobility of oyster hemocytes, McCormick-Ray and Howard (1991), who video-recorded and tracked the locomotive rates of *C. virginica* hemocytes, reported that only 68% of the tracked granulocytes were mobile, with the "basophilic granulocytes" being the most active. Granulocytes were almost four times more active than hyalinocytes. McCormick-Ray and Howard (1991) also reported seasonal differences in the percentages of mobility among oyster hemocyte types. Specifically, "nonlymphoid" hyalinocytes are most mobile in February, followed by March and May, and "lymphoid" hyalinocytes are most mobile in March, followed by January. The highest percentage of mobile granulocytes was reported in March, then January and February. These differences support the finding by Fisher et al. (1989) that cell mobility is correlated with the time (month) at which the cells are examined and also suggest functional differences between the categories of hemocytes.

Number of Hemocytes

Because of the open circulatory system in oysters and the influx and efflux of hemocytes and serum from sinuses and tissues, large fluctuations in both the number of cells and concentrations of serum components (e.g., proteins, carbohydrates, salts, ions, etc.) are to be expected. For example, Cheng (1988) reported that the total hemocyte count (± 1 SD) in *C. virginica* varied from about 1354 ± 115 to 1548 ± 120 ml^{-1} during a 2-week period. Consequently, the establishment of baseline counts of hemocytes circulating in oysters is difficult. Nevertheless, some data pertaining to the ratios between granulocytes and hyalinocytes are available. Foley and Cheng (1975) reported that granulocytes (i.e., typical granulocytes plus "fibrocytes," or spent granulocytes, see p. 302) comprised about 87.5% and hyalinocytes comprised about 12.9% of the total number of hemocytes in *C. virginica* that they examined. McCormick-Ray and Howard (1991) reported that granulocytes comprised 61% and hyalinocytes comprised 39% of the total number of cells in *C. virginica* that they examined. The apparent discrepancy is probably due to differences in the ages, sizes, physiological states, ambient environmental (including temperature) and other factors that influence the number of circulating cells in oysters. It is known, for example, that the number of hemocytes is influenced by the heart rate and pressure, which in turn are influenced by the ambient temperature (Feng 1965); furthermore *in vivo* exposure to 1 ppm of Cd^{2+} stimulates hemopoiesis, results in a reduction in the percentage of hyalinocytes, and causes an elevation in the percentage of hyalinocytes after 1 and 2 weeks (Cheng 1988).

Functions of Hemocytes

Oyster hemocytes are known to be involved in several vital functions: wound repair, shell repair, nutrient digestion and transport, excretion, and internal defense. Brief reviews of these functions follow.

Wound Repair

The role of bivalve, especially oyster, hemocytes in wound repair has been expertly reviewed by Sparks (1972) and need not be duplicated here in detail. Before commenting on wound repair, I consider here the clumping phenomenon as it pertains to oyster hemocytes. Aggregation of bivalve hemocytes to form clumps was reported initially by Drew (1910), who studied the cells of the cockle, *Cardium norvegicum*, by Dundee (1953), who examined several species of freshwater bivalves, and by Narain (1972), who examined the cells of the freshwater bivalve *Lamellidens corrianus*. Foley and Cheng (1972) reported that it is primarily granulocytes that form clumps in *C. virginica*; furthermore, it is only cells freshly drawn from the adductor muscle sinus that form aggregates, which include from 3 to 5 up to 100 to 200 cells. If the preparation is permitted to stand, there is cell spreading and exomigration from each cluster as described previously. The clumping of bivalve hemocytes is physiologically different from the clotting of vertebrate blood in that a fibrinogen-thrombin-fibrin system is absent in molluscs (Narain 1972).

Hemocyte clumping in bivalves is intimately involved in the wound healing process. Specifically, clumped hemocytes have been reported to delineate and arrest hemorrhage (Dakin 1909; Drew 1910; Goodrich 1919; Orton 1923; des Voigne and Sparks 1968, 1969; Ruddell 1971a, b, c).

Wound repair in oysters (and other bivalves) involves five stages: (1) the initial stage involves the infiltration of the wounded area by large numbers of hemocytes; (2) the hemocytes aggregate to form a plug as well as delineate the wound; (3) healing proceeds from the interior of the lesion toward the surface, with the replacement cells, which are fusiform, having differentiated from clumped hemocytes – in *C. gigas*, the wound channel is effectively plugged in 144 h after experimental wounding (des Voigne and Sparks 1968); (4) collagen is deposited between the cells forming the plug; however, in time, both the hemocytes and collagen are replaced by Leydig cells (= vesicular connective tissue cells); (5) phagocytic hemocytes, most probably granulocytes, eventually infiltrate and endocytose the necrotic cellular debris.

Events subsequent to the above, involving differentiation of hemocytes or migration of epithelial cells, are beyond the scope of this discussion (see Cheng 1981 and Sparks 1985 for reviews).

Shell Repair

A detailed study of shell repair in oysters has not been published (see Carriker, Chapter 3). However, the process has been examined in several other bivalve species and reviewed by Wilbur (1964), Abolins-Krogis (1968), and Cheng (1981). In brief, shell regeneration involves (1) initiation of stimulating signal(s) resulting from injury (including experimental removal of a portion of the shell); (2) mobilization of calcium and other molecules (proteins, etc.) from different regions of the organism; (3) transport of calcium and other molecules to the area of repair; and (4) localized deposition of organic matrix and calcium carbonate. The major role of hemocytes in this series of events is the transport of calcium and proteins.

Nutrient Digestion and Transport

The role of molluscan hemocytes in nutrient digestion and transport, in view of recent findings, cannot be considered an isolated function. It is commonly so intimately associated with internal defense that, in many ways, the two become indistinguishable. Consequently, details of intracellular digestion will be considered below under "Internal Defense" and only the general framework is considered here. The role of hemocytes in digestion has been reviewed extensively by Takatsuki (1934a,b), Wagge (1955), Owen (1966), Purchon (1968), Narain (1973), and Cheng (1981) and need not be reconsidered in detail.

Digestion in bivalves is both extra- and intracellular (see Langdon and Newell, Chapter 6). Extracellular digestion is effected in the stomach by enzymes released from dissolution of the crystalline style. Most authorities agree with Yonge (1937, 1946) that intracellular digestion is more important. This type of digestion occurs within two categories of cells: hemocytes and digestive cells of the digestive diverticula. Recall that bivalves have an open circulatory system and hemocytes occur not only within the heart, hemolymph vessels, and sinuses but also are found migrating through tissues. Some migrate into the lumen of the alimentary tract from between the cells of the lining epithelium. These pinocytose soluble nutrients (some already partially digested by enzymes of style origin) and phagocytose particulate foodstuffs. Once material is endocytosed, digestion commences, and concurrently, the hemocytes pass back into the deep tissues of the body and transport nutrients to various tissues (Yonge 1926).

In a very significant study, Feng et al. (1977) reported finding carotenoids, specifically, flavenoids, b-carotene, canthaxanthin, and other unidentified xanthophylls, in hemocytes of *C. gigas* (and the bivalve, *Mytilus coruscus*). The presence of these pigments, which can only be synthesized by plants, in bivalve hemocytes indicates that these molluscs may have acquired them by phagocytosing pigment-bearing unicellular algae in their diet. It is especially significant that Feng et al. (1977) found carotenoids in hemocytes bled from the adductor muscle sinus. This supports Yonge's (1926) contention that food-particle-laden hemocytes in the lumen of the alimentary tract traverse the lining epithelium and migrate into the deep tissues. Relative to this phenomenon, Feng et al. (1977) raised an interesting point based on reports by Yonge (1926), Stauber (1950), Tripp (1960), and Feng et al. (1977). They concluded that there is a two-way traffic of bivalve hemocytes across the epithelial lining of the alimentary tract and the movement of cells is not random.

What are the mechanisms governing the direction of hemocyte migration? Stauber (1950), Tripp (1960), and others have reported that hemocytes laden with noninjurious foreign materials, e.g., India ink particles, bacterial spores, migrate through the alimentary wall to the lumen. Conversely, Yonge (1926) and Feng et al. (1977) found cells laden with materials derived from food to move from the lumen through the epithelium into deep tissues. Thus, it may be that the directional migration of hemocytes is in some way regulated by the nature of the endocytosed materials. In other words, if the endocytosed material is of little or no nutritional value, the hemocytes migrate out of the body; however, if the material represents a source of nourishment, then the cells migrate into the deeper tissues.

The second site of intracellular digestion in bivalves is the digestive diverticula. The end products of digestion in the diverticular cells are believed to be transferred to hemocytes and serum, which transport them to various tissues of the body.

Excretion

Although the function of the excretory system of bivalves has been studied extensively (see reviews by Grassé 1960; Galtsoff 1964; Martin and Harrison 1966), relatively little is known about the role of hemocytes. Although Orton (1923) reported the elimination of metals as a result of endocytosis by hemocytes and the subsequent elimination of the metal-laden hemocytes, it was not ascertained whether the nephridial tubules were the main sites of excretion. This also holds true for the removal of necrotic hemocytes in *C. gigas* (Sparks and Pauley 1964).

As discussed earlier, serous cells are known to play a role in excretion (for reviews, see White 1942; Cheng 1981). In brief, serous cells not only remove degradation products of dead or moribund parasites and the metabolic by-products of successful parasites but also extract acids from the hemolymph which they carry to the kidney (organ of Bojanus) via the ciliated reno-pericardial channels (White 1942).

Internal Defense

In recent years, much of the research on oyster hemocytes has been directed at understanding their role in internal defense against exogenous biotic and abiotic materials. With the increased popularity of comparative immunology as a subdiscipline, the study of molluscan internal defense mechanisms has been defined as one aspect of immunology, with the direct roles of hemocytes being considered as one form of cell-mediated immunity.

The term "immunity" may not be appropriately applied to molluscs because their defense system is not characterized by the occurrence of amnesis, the synthesis of immunoglobulins, etc. In recent years, however, the occurrence of specificity at the molecular level has been recognized (Cheng 1985, 1986). Nevertheless, there is no doubt that there are humoral and cell-mediated factors that serve protective roles and hence "immunity" is being employed here in the broadest sense.

Cellular, or cell-mediated, immunity in oysters can be conveniently described as phagocytosis or encapsulation; however, as Cheng and Rifkin (1970) pointed out, encapsulation may reflect aborted attempts at phagocytosis. In view of this, the phases of phagocytosis are considered here.

Modern studies aimed at understanding cellular immunity in oysters commenced with Stauber (1950) who traced the ultimate disposition of India ink particles introduced experimentally into *C. virginica.* Critical, detailed reviews of the fates of a variety of experimentally introduced foreign materials endocytosed by molluscan hemocytes have been published since then (e.g., Cheng 1967; Feng 1967). Briefly, it is known that digestible particles and macromolecules are degraded within oyster hemocytes (Tripp 1958, 1960; Feng 1959, 1965) whereas indigestible particles and macromolecules are voided by the migration of foreign material-laden phagocytes across certain epithelial borders and their shedding from the oyster (Stauber 1950; Tripp 1960; Feng 1965). Not all invading organisms are endocytosed. For example, the oyster pathogen *Haplosporidium nelsoni* is usually not endocytosed. Similarly, the flagellate *Hexamita nelsoni* and *Staphylococcus aureus* phage 80 introduced experimentally into *C. virginica* induce little or no phagocytosis (Feng 1966; Canzonier, quoted in Feng 1967; Feng and Stauber 1968).

Although most investigators believed that oyster granulocytes are the most active phagocytes, it was not until Foley and Cheng (1975) performed quantitative studies on hemocytes of *C. virginica* (and *M. mercenaria*) that it was ascertained that all cells are phagocytic; however, of these, granulocytes are considerably more active. Specifically, when the percentages of "primary fibrocytes" and "secondary fibrocytes," which are now believed to be degranulated granulocytes that have phagocytosed bacteria, were added to the percentage of granulocytes that had engulfed bacteria, 87.3% of granulocytes, as compared to 12.3% of hyalinocytes, were associated with experimentally-introduced *Staphylococcus aureus,* and 83.5% of granulocytes, as compared to 16.8% of hyalinocytes, were associated with experimentally introduced *Escherichia coli.* On the other hand, Farley (1968) and Ruddell (1969) suggested that agranular cells are more phagocytically active. However, they may have interpreted granulocytes at a later stage of the phagocytosis-intracellular degradation continuum to be hyalinocytes (Cheng 1981).

It is generally accepted that phagocytosis can be separated into four phases: (1) attraction between phagocyte and non-self material, (2) surface attachment, (3) internalization, and (4) in most instances, intracellular degradation. These phases are described in detail in the following four sections.

Attraction between Phagocytes and Non-self Material. The coming together of oyster phagocytes and non-self material prior to contact could occur either randomly or reflect non-random attraction. As a result of examining histological sections of oysters naturally or experimentally infected with microbial and metazoan/protistan invaders, positive chemotactic reaction, i.e., attraction to a chemical stimulatory gradient, was suspected. This led to the report that *C. virginica* hemocytes are attracted by the metacercarial cyst of the digenetic trematode *Himasthla quissetensis* (Cheng et al. 1974) and the report that the cells are also attracted by *Micrococcus varians* (= *Staphylococcus latus*) (Cheng and Rudo 1976b). Subsequently, Cheng and Howland (1979) tested two Gram-positive bacteria (*Bacillus megaterium* and *Micrococcus varians*) and two Gram-negative bacteria (*E. coli* and *Vibrio parahaemolyticus*) and showed that oyster hemocytes are attracted to live *E. coli*, *B. megaterium*, and *M. varians* but not to heat-killed bacteria. Furthermore, oyster cells are not attracted to either live or heat-killed *V. parahaemolyticus*. Thus, the chemoattractant may be a molecule emitted by living vegetative cells of certain Gram-positive as well as Gram-negative bacteria. Also, in the case of positive chemotaxis, it has been postulated that a recognition receptor for the chemoattractant exists on the hemocyte surface (Vasta et al. 1982).

Vibrio parahaemolyticus, which is nonattractive to *C. virginica* hemocytes, is a known pathogen of marine bivalves, especially larvae (for review, see Sindermann 1990). It has been proposed that the characteristic of not attracting host hemocytes may be important for pathogen survival (Cheng and Howland 1979).

The apparent non-recognition of certain invaders need not be exclusively related to the absence of chemotactic attraction between them and hemocytes. It may reflect antigen sharing between molluscan hemocytes and protistan (Kanaley and Ford 1990) and metazoan parasites (Yoshino and Cheng 1978).

In addition to the above, Tripp (1975) hypothesized the occurrence of an "all purpose" hemolymph protein that coats and tags non-self entities. However, as Feng (1988) pointed out, coating with a host protein can either facilitate or inhibit their recognition. Feng and Barja (1987) speculated that the lack of host responses, e.g., phagocytosis, could be independent of self/non-self recognition. Such a pattern could reflect the presence of factors that inhibit membrane synthesis and of receptor-blocking substances of parasite origin.

Because *C. virginica* hemocytes are attracted to *E. coli* and *B. megaterium*, which are Gram-positive and Gram-negative, respectively, Howland and Cheng (1982) proceeded to partially identify the bacterial chemoattractants. They reported that hemocytes are attracted to proteins of approximately 10,000 daltons that are associated with the cell wall of *B. megaterium* and the cell envelope of *E. coli*. Also, Cheng and Howland (1982) reported that chemotactic attraction of *C. virginica* hemocytes to *B. megaterium* is dependent on an intact cytoskeletal system. Chemotaxis is inhibited by pretreatment of hemocytes with such pharmacologic agents as colchicine, which inhibits the assembly of microtubules by binding to monomeric tubulin and preventing its polymerization into microtubules, and cytochalasin B, which reduces the rate of actin polymerization into microfilaments.

Surface Attachment. The binding of non-self material to a molluscan phagocyte represents the second phase of cellular response. In view of what is known about cell surface binding in both vertebrates and invertebrates, this is usually not a random, nonspecific phenomenon. As has been reviewed earlier (Cheng 1984b; Cheng et al. 1984), the currently popular concept is that naturally occurring lectins serve as bridges for binding the insulting agent to the molluscan phagocyte. Such lectins may occur in serum (e.g., Prowse and Tate 1969; Anderson and Good 1976; Renwrantz 1981) or are integrally associated with the surface membrane of phagocytes (Vasta et al. 1982, 1984; Cheng et al. 1984).

In the case of *C. virginica*, Vasta et al. (1982) demonstrated a lectin associated with the plasma membrane of hemocytes by using a microhemagglu-

tination assay. The plasma membrane association of this lectin was shown by its copurification with the plasma membrane fraction of disrupted hemocytes using sucrose density gradient centrifugation, and also by the binding of ^{125}I-labelled glycoproteins to intact hemocytes at 4°C. Based on agglutinating specificity for a range of untreated and enzyme-treated vertebrate erythrocytes, along with hemaglutination-inhibition assays and cross-adsorption tests, it was found that there are also two serum (soluble) lectins, each having a distinct serological agglutination specificity, and that the hemocyte membrane-associated lectin has a specificity that is identical with one of these two serum lectins. Vasta et al. (1982) proposed that the hemocyte membrane-associated lectin may be a true integral membrane protein, and therefore may function as a membrane receptor in non-self recognition by oyster hemocytes.

Vasta et al. (1984) took advantage of the finding that the hemocyte membrane lectin has an identical specificity with one of the two serum lectins: by employing antisera produced against this serum lectin, and using immunofluoresence, they showed that the membrane lectin is situated on the external surface of the cell membrane. Furthermore, the antisera block the binding of hemocyte microsomes to protease-treated vertebrate erythrocytes, thus confirming that the hemocyte membrane lectin is serologically related to the serum lectin. This serum lectin has an apparent mass of 34,000 daltons. Also, it was demonstrated by employing flow cytometry that the distribution of the cell surface lectin is heterogeneous throughout the hemocyte population, but no discrete cell subpopulations could be identified.

In addition to binding phagocytes to non-self materials, molluscan lectins also serve as opsonins, i.e., coat the foreign particles or cells so as to enhance their ingestion by phagocytes (Anderson and Good 1976; Renwrantz 1983; Coombe et al. 1984; Vasta and Marchalonis 1984; Olafsen 1988). Tripp (1966) demonstrated that the serum of *C. virginica*, which agglutinates rabbit erythrocytes, also enhances the *in vitro* uptake of these erythrocytes by oyster hemocytes. Naturally occurring lectins of several other invertebrates, including molluscs, are known to be opsonic (see Olafson 1988 for review).

Fisher and DiNuzzo (1991) reported that among the sera of six species of marine molluscs tested, that of *C. virginica* agglutinates 43 of 94 bacterial isolates (belonging to 15 genera and 36 species). This observation suggests that the 43 bacterial isolates share saccharides on their surfaces with which a serum lectin of *C. virginica* has an affinity or that there is more than one serum lectin in the oyster and each has an affinity for a sugar common to more than one bacterial isolate. That the latter is possible is supported by the finding by Vasta et al. (1984) that there are least two different lectins in the serum of *C. virginica.*

In view of the concept that lectins bind oyster phagocytes to non-self materials that are eventually endocytosed, it is of interest to note that Kanaley and Ford (1990) reported that both *C. virginica* hemocytes and the plasmodial stages of *H. nelsoni* are agglutinated by concanavalin A and wheat germ agglutinin; however, in addition, hemocytes are agglutinated with *Phaseolus vulgaris* (PHA) kidney bean lectin and *Limulus polyphemus* (LPA) horseshoe crab lectin. These results indicate that oyster hemocytes possess surface receptors resembling N-acetyl-D-glucosamine and α-methylmannopyranoside and that *H. nelsoni* plasmodia possess surface receptors resembling N-acetyl-D-glucosamine, α-methylmannopyranoside, glucose, sucrose, and mannose. Kanaley and Ford (1990) also reported antigenic similarities between surface receptors on oyster hemocytes and those of *H. nelsoni* plasmodia, which may explain the failure of hemocytes to phagocytose this parasite (i.e., the hemocytes do not recognize the parasites as foreign because they share common surface components). Kanaley and Ford (1990) noted that seasonal variability occurs in Con A agglutination titers.

Relative to lectin-binding by oyster hemocytes, Cheng et al. (1993), by employing eight lectins (ConA, *Tetragonolobus purpureas, Limulus polyphemus, Dilichos biflorus, Sambucas nigra, Glycine max, Triticum vulgaris, Lathyrus odoratus*) and the known inhibiting sugar residues, demonstrated that the following saccharides occur on hemocytes of *C. virginica* from Apalachicola Bay, Florida, and Galveston Bay, Texas: N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, methyl-α-D-mannopyranoside, D(+)-glucose, sucrose, D(+)-mannose, α-methyl-D-galactoside, β-D(-)-fructose,

L(-)-fucose, N-acetylneuraminic acid, and D(+)-galactose. D-glucuronic acid only occurred on oyster cells from Apalachicola Bay. An unidentified sugar that is neither D(+)-glucose nor D(+)-mannose, the usual inhibiting sugars for the *L. odoratus* lectin, occurred on hemocytes from both collection sites. This saccharide is called "lathyrose" until it can be identified. The quantitative and qualitative differences in the saccharidal constituents on the surfaces of hemocytes from oysters from the two sites are attributed to strain differences.

Because lathyrose occurs on hemocytes of oysters from the Gulf of Mexico, where *H. nelsoni* has never been reported, and on hemocytes of South Carolina and Chesapeake Bay oysters that were free of *H. nelsoni*, Cheng (1994) has proposed that the occurrence of hemocyte membrane lathyrose may serve as a marker for resistance to *H. nelsoni*. Lathyrose apparently also occurs on the surfaces of intramolluscan stages of *P. marinus* because the presence of this parasite partially inhibits the agglutination of hemocytes of oysters resistant to *H. nelsoni* (Cheng 1994).

As Vasta et al. (1984) pointed out, the occurrence of lectins on *C. virginica* hemocytes and in serum is somewhat analogous to the vertebrate immunoglobulin system in that these are both soluble immunglobulin molecules and membrane-bound immunoglobulins present in subsets of immunocompetent cells. However, two major differences exist. Lectins are thought to be constitutive (of nonimmune origin) whereas antibodies are induced, and lectins do not portray the heterogeneity in specificity observed in the immunoglobulin system. Nonetheless, invertebrate humoral and membrane lectins may still represent a primitive nonself recognition system based on carbohydrate binding molecules.

Endocytosis. There have been no reported studies of endocytosis of foreign materials by phagocytes of any mollusc at the biophysical and molecular levels. However, at least three morphologically distinguishable processes have been reported for *C. virginica* granulocytes. Specifically, Bang (1961) reported the first type wherein motile bacteria initially become adhered to the surface of the molluscan cell, commonly on the surface of filopodia. Subsequently, they are taken into the ectoplasm by gliding along the filopodia and becoming enclosed in a phagosome (Fig. 10). This process is now known as the "Bang mechanism." The second type was discovered as a result of studying the uptake of *Bacillus megaterium* by *C. virginica* granulocytes (Cheng 1975). This endocytosis mechanism involves the formation of an invagination on the cell surface and the bacterium is endocytosed into a vesicle (Fig. 10). No filopodia is involved. The third type of endocytosis was initially reported by Renwrantz et al. (1979). They studied the uptake of rat erythrocytes by *C. virginica* hemocytes *in vitro* and found that both granulocytes and hyalinocytes (primarily the former) commonly endocytose blood cells by producing a funnel-like pseudopod through which the foreign cell glides into a phagosome in the ectoplasm (Fig. 10). The occurrence of this mechanism with *C. virginica* phagocytes has been confirmed by Hinsch and Hunte (1990).

As stated, the details of endocytosis at the biophysical and molecular levels remain to be elucidated and it is not known what governs the mechanism (or type) of endocytosis employed. The hypothesis has been advanced that molecular specificity is involved (Cheng 1985, 1986).

A very popular model for explaining internalization is the "zipper mechanism" involving interaction between a ligand (or some other molecule) on the surface of the foreign particle or organism with a complementary receptor for the ligand. This is followed by the protrusion of a pseudopodium (or pseudopodia) that engulfs the invader into a phagosome (Silverstein 1977). Although the "zipper mechanism" is a reasonable general model, there are, as outlined above, at least three different, distinguishable endocytotic processes in the case of oyster granulocytes.

Intracellular Degradation. Most foreign materials that become endocytosed are degraded intracellularly. The morphological manifestation of this process at the light microscope level was recorded by Tripp (1958, 1960). Since then, the processes involved have been somewhat clarified as a result of electron microscopical and biochemical studies. The basic process is essentially the same whether it is foodstuffs, engulfed foreign molecules, or organisms that are being digest-

ed. Because granulocytes are more phagocytic than hyalinocytes (Foley and Cheng 1975) and one of the major differences between these types of cells is the occurrence of large numbers of cytoplasmic granules, it was proposed that resolution of the nature of these granules may provide some clues to the phagocytic process, including intracellular degradation (Yoshino and Cheng 1976a).

Based on present information, the cytoplasmic granules of the bivalves *M. mercenaria*, *Mya arenaria*,

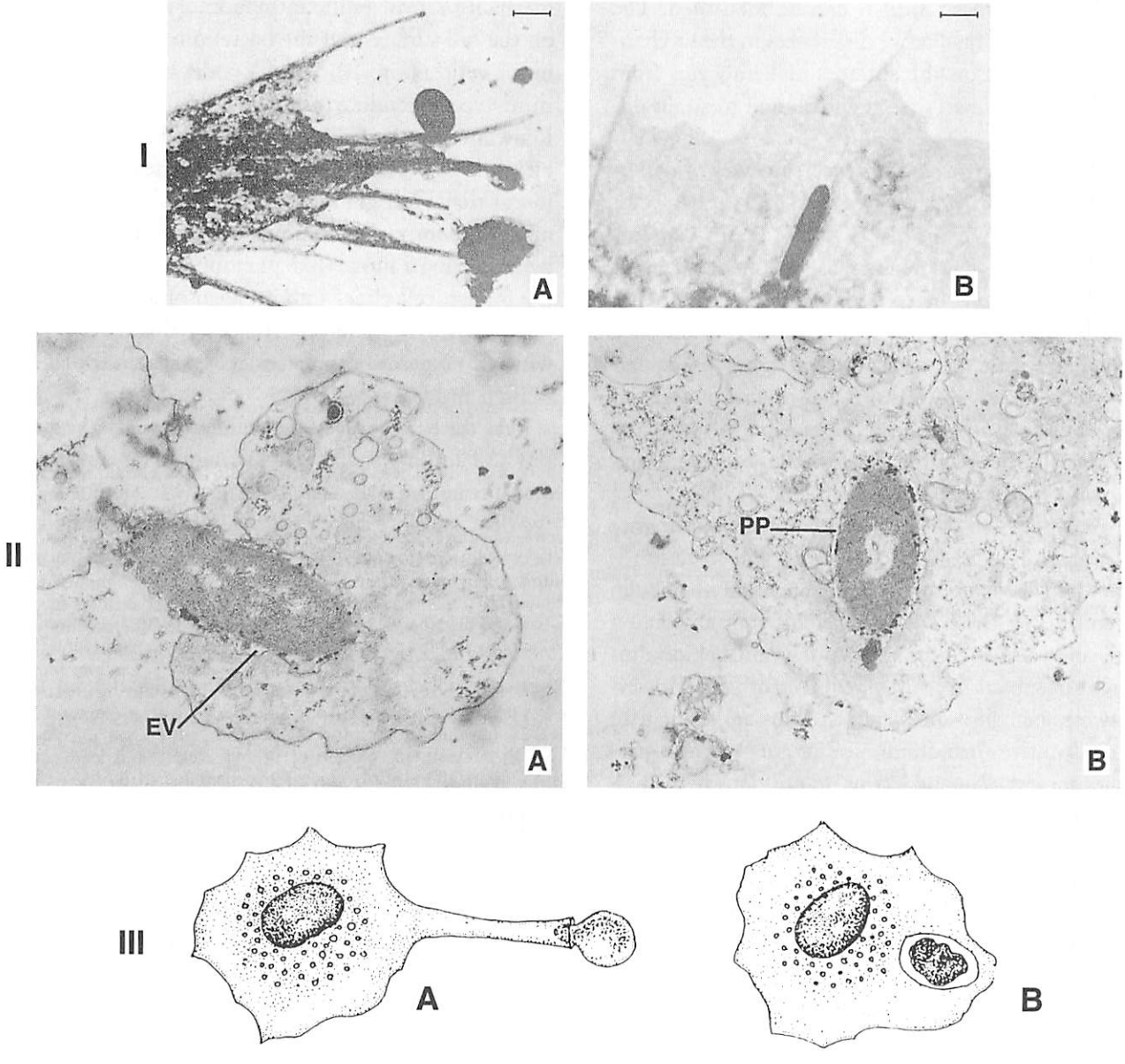

Figure 10. Three endocytotic mechanisms employed by *Crassostrea virginica* hemocytes. (IA) Electron micrograph of beginning of phagocytosis of bacterium by the Bang mechanism; × 14,000. (IB) Endocytosed bacterium in ectoplasm of phagocyte; × 8600. IA and IB after Bang (1961). (IIA) Endocytosis of *Bacillus megaterium* without aid of filopodium. [EV] endocytotic vesicle. (IIB) Endocytosed bacterium in primary phagosome [PP] × 20,000. IIA and IIB after Cheng (1975). (IIIA) Uptake of rat erythrocyte through "funnel" formed by granulocyte. (IIIB) Endocytosed erythrocyte in phagosome. IIIA and IIIB after Cheng (1981).

and *Spisula solidissima* are true lysosomes (Yoshino and Cheng 1976a; Cheng 1981). As in all eukaryotic cells, these are membrane-lined organelles in which acid hydrolases are packaged and stored. Thus, these organelles are analogous to the granules in mammalian polymorphonuclear and monocytic leucocytes. The functions of these molluscan hemocytic lysosomes in intracellular and extracellular digestion have been considered by Cheng (1981) and Mohandas (1985).

From what is known, the intracellular digestion process in eastern oysters (Fig. 11) is somewhat different from that outlined above for other bivalve species. As reported by Cheng and Cali (1974) and Cheng (1975), who traced the fate of bacteria endocytosed by *C. virginica* granulocytes with electron microscopy, the degradation of bacteria commences in initial (or primary) phagosomes where the appearance of concentric lamellae occurs. Then the partially degraded bacteria are transferred to secondary phagosomes where remnants of the bacteria are subjected to further degradation. Again, digestive lamellae are synthesized and deposited around the bacteria being digested. Eventually, the digestive lamellae disappear, and the exogenous material (presumably the carbohydrate constituents that had been degraded to a hexose, probably glucose) is polymerized into glycogen. Concurrent with the appearance of glycogen granules, primarily as rosettes, in the lumen of the phagosome, the complex phagosomal wall commences to fragment (Fig. 12) and eventually disappears. This process results in the deposition of clumps of glycogen granules free in the cytoplasm (Fig. 13). The glycogen granules eventually fuse into masses and are subsequently discharged into serum in packets enveloped by the plasma membrane of the granulocyte.

Cheng (1975) demonstrated fusion of lysosomes with what have been designated "secondary lysosomes"; however, the source of the enzymes responsible for the initiation of digestion within the initial or primary phagosome remains undetermined. The possibility exists that lysosomal fusion with initial phagosomes also occurs.

The membrane-bound glycogen is released into serum when the membrane is degraded by lysosomal enzymes (see pp. 323). Subsequently, the glycogen discharged from cells is, by some yet undetermined pathway, degraded to glucose, which becomes distributed by the hemolymph to various cells in the body.

The processes summarized above appear to be supported by the results of Cheng and Rudo (1976a), who injected ^{14}C-labelled *Bacillus megaterium* into *C. virginica* followed by extraction of glycogen from hemocytes, sera, and body tissues at several time intervals. It was found that ^{14}C is first detected in glycogen extracted from hemocytes and later in serum and tissues. Earlier, Cheng (1975) reported the results of a comparable experiment in which specimens of *C. virginica* were not exposed (controls) or were exposed to 3×10^8 *E. coli in vivo* for 2 h. Subsequently, the amount of glycogen present in the hemolymph of control oysters was 32.87 ± 1.34 μg ml^{-1} of hemolymph but was 39.73 ± 1.26 μg ml^{-1} in the experimental oysters, a statistically significant difference. Similarly, Rodrick and Ulrich (1984) reported a significant rise in hemolymph glycogen in the bivalves *C. virginica*, *Mercenaria campechiensis*, and *Anadara ovalis* at 1 h post-challenge *in vivo* with *E. coli* and *Vibrio anguillarum*.

It may be somewhat surprising that there appear to be two sets of phagosomes (initial and secondary) in *C. virginica* granulocytes. Feng et al. (1971), Cheng and Cali (1974), and Cheng et al. (1974) did determine, however, that the cytoplasmic granules and phagosomes of *C. virginica* granulocytes are structurally different from those in hemocytes of other bivalves. Specifically, most of the cytoplasmic granules, which are true membrane-delimited lysosomes in the granulocytes of other species of molluscs (Yoshino and Cheng 1976a), include a thick layered membrane wall. In fact, as stated earlier, Feng et al. (1971) classified these unusual organelles into three types. Also, Cheng et al. (1974), based on the reconstruction of sections observed with a transmission electron microscope, described each granule to be elongate ovoid, with the cortex being thicker at the two ends. The surface overlying the cortex consists of a unit membrane. This membrane may be lifted from the cortex so that a lucent space is apparent between the surface membrane and cortex (Fig. 14). The core of each granule is usually lucent.

The phagosomes of oyster phagocytes, at least the secondary phagosomes, are also different from those of other molluscs, e.g., those of *M. mercenaria* (see Mohandas 1985). Those of *C. virginica* have a considerably more electron-dense outer surface comprised of a thick outer wall, an electron lucent middle zone, and an inner layer of medium electron density. As depicted in Fig. 12, when the phagosomal wall commences to disintegrate after the appearance of glycogen granules, the units of the fragmented wall appear as hollow tubules, some with closed terminals.

Energy Requirements

Even though endocytosis, intracellular digestion, and associated processes require energy, there is no increase in oxygen consumption by *M. mercenaria* hemo-

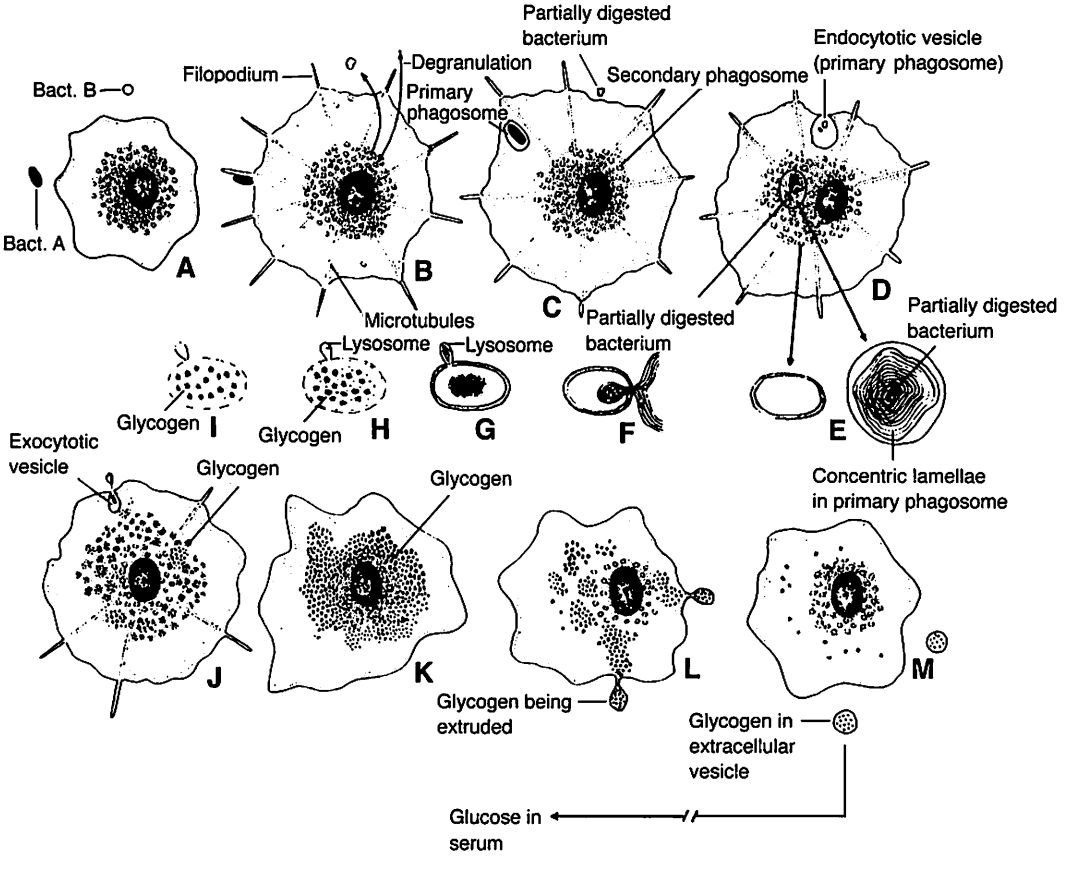

Figure 11. Sequence of events that occur during phagocytosis and intracellular degradation of bacteria by *Crassostrea virginica* granulocytes. (A) Granulocyte in proximity of bacteria A and B. (B) Bacterium A becomes attached to filopod whereas bacterium B is altered by enzymes released from granulocyte during degranulation. (C) Bacterium A is within primary phagosome of granulocyte. (D) Bacterium B is endocytosed and digestion of bacterium A has begun within primary phagosome. (E) Formation of digestive lamellae around bacterium in primary phagosome; secondary phagosome in vicinity. (F) Transfer of partially digested bacterium from primary to secondary phagosome. (G) Lysosome fused with and discharging enzymes into secondary phagosome. (H) Glycogen synthesized from sugar constituents of degraded bacterium; phagosomal wall disintegrating. (I) Phagosomal wall disintegrating. (J). Discharge of nondegradable remnants of bacterium via exocytotic vesicle (=residual body); accumulation of glycogen in granulocyte cytoplasm. (K) Massing of glycogen in cell and disappearance of filopodia. (L) Glycogen in process of being discharged into serum in packets. (M) Glycogen discharged. After Cheng (1975).

cytes actively phagocytosing *B. megaterium* (Cheng 1976). In *M. mercenaria*, both glucose and glycogen are used and lactate is produced (Cheng 1976); however, de Zwaan (1977, 1983) claimed that lactate is not produced in bivalves. The absence of increased oxygen uptake, the utilization of glycogen and glucose, and the reported production of lactate indicate that glycolysis is the energy-producing pathway in *M. mercenaria*. It remains to be determined whether a similar pattern occurs in *C. virginica* and other oysters.

The conclusion that glycolysis is the energy-providing pathway is strengthened by the fact that KCN does not inhibit phagocytosis (Cheng 1976). Also, it has been reported that the nitro-blue tetrazolium reduction characteristic of mammalian phagocytes is absent in *M. mercenaria* hemocytes, and the myeloperoxidase-H_2O_2-halide antimicrobial system of mammalian phagocytes is also absent (Cheng 1976).

Alvarez and Friedl (1990) reported that no difference exists between the percentages of endocytosis of polystyrene beads by *C. virginica* hemocytes under

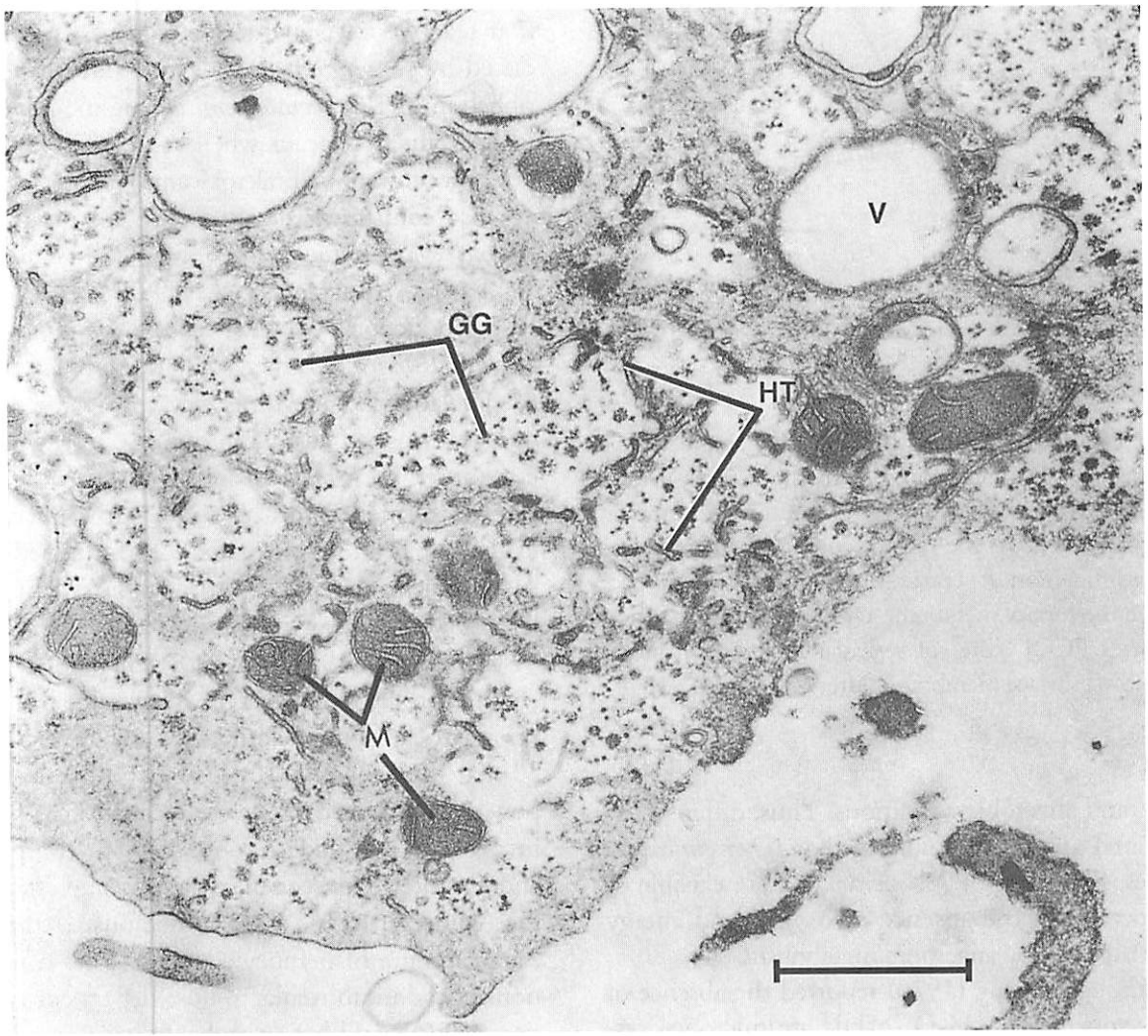

Figure 12. Electron micrograph of a portion of *Crassostrea virginica* granulocyte showing glycogen granules [GG] in lumina of secondary phagosomes, the complex walls of which have begun to disintegrate. [HT] hollow tubules, [M] mitochondria, [V] vesicle. Bar = 1 μm. After Cheng et al. (1974).

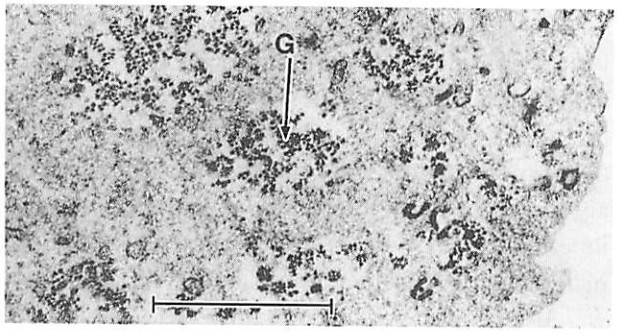

Figure 13. Electron micrograph showing aggregates of glycogen [G] in the cytoplasm of a *Crassostrea virginica* granulocyte. Bar = 1 μm. Note absence of enveloping secondary phagosomal wall. × 40,000. After Cheng and Cali (1974).

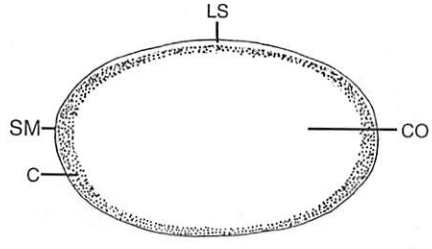

Figure 14. Reconstructed drawing of a single vesicle (granule of light microscopy) from the cytoplasm of a *Crassostrea virginica* granulocyte. Note thicker cortex at the two ends and at the region where the surface membrane is lifted from the cortex, thus creating a lucent space between the surface membrane and the underlying cortex. [C] cortex, [CO] "core" of vesicular lumen, [LS] lucent space, [SM] surface membrane. After Cheng et al. (1974).

aerobic and anaerobic conditions. Thus, different experimental approaches indicate that *C. virginica* hemocytes, like those of *M. mercenaria*, are capable of phagocytosis in the absence of oxygen and energy can be supplied by anaerobic metabolism.

Although Cheng (1976) reported the absence of the myeloperoxidase-H_2O_2-halide antimicrobial system in *M. mercenaria*, the occurrence and roles of oxygen metabolites in molluscs are unresolved issues. The reactive oxygen metabolites O^- and H_2O_2 have been reported in hemocytes of the scallop *Patinopecten yessoensis* (Nakamura et al. 1985; Mori et al. 1990), the freshwater snail *Lymnaea stagnalis* (Dikkeboom et al. 1987, 1988a, b), and a second freshwater gastropod, *Biomphalaria glabrata*, especially those infected with schistosomes (Shozawa et al. 1989; Connors and Yoshino 1990; Connors et al. 1991). Also, Granath and Yoshino (1983) and Gelder and Moore (1986), by employing cytochemical techniques, reported the occurrence of peroxidases in hemocytes of *B. glabrata* and *M. mercenaria*, respectively. The effectiveness of these oxygen metabolites as antimicrobial and antiparasiticidal agents *in vivo* remains unknown. More recently, Anderson (1994) reported that reactive oxygen intermediates (ROI) are produced by *C. virginica* hemocytes (and those of the ribbed mussel *Geukensia demissa*) and modulations of ROI production occur when the hemocytes are exposed to environmental toxicants or disease agents. He also confirmed that ROIs are not produced by *M. mercenaria* hemocytes.

Earlier, Anderson et al. (1992c) reported that ROI production by hemocytes of *C. virginica* infected with *P. marinus* is increased and the total number of circulating hemocytes is elevated, i.e., occurrence of leucocytosis. Thus, advanced cases of Dermo disease caused by *P. marinus* are characterized by hemocyte recruitment and activation, with concomitant exuberant production of hemocyte-derived ROIs. Also, Anderson et al. (1992a) demonstrated that low levels of ROIs occur in about 40% of unchallenged oyster hemocytes; however, ROI generation is greatly elevated in cells that had phagocytosed heat-killed yeast particles at temperatures >21°C. It was concluded that the generation of ROIs is probably a component of the internal defense mechanisms of oysters involved in cytotoxic responses to microorganisms and parasites. Consequently, it is of interest to note that Anderson et al. (1992b) demonstrated that *in vitro* exposure of hemocytes to sublethal concentrations of cadmium results in dose-dependent suppression of ROIs and hence probably impairs the ability of hemocytes to kill microorganisms. The presence of autologous serum, i.e., serum from the same oyster, in the culture medium protects the cells from the ROI-suppressive effects of cadmium.

Hemocytes and Humoral Factors

Although the internal defense mechanisms of oysters (and other molluscs) can be conveniently separated into cell-mediated and humoral types, it has become increasingly apparent that the two are interrelated. Consequently, any review of the functional roles of molluscan hemocytes must include at least an abbreviated account of humoral factors and, where known, the relationship between hemocytes and humoral factors. The following is presented with these considerations in mind. Recent detailed accounts of molluscan humoral factors have been presented by Chu (1988) and Cheng and Combes (1990).

Agglutinins

Hemagglutinins have been reported from *C. virginica* hemolymph (Li and Fleming 1967). Because these have not been isolated and characterized, it is uncertain whether they are true lectins, i.e., specialized protein complexes that bind to specific saccharides and cause the agglutination of cells or molecules by glycosyl moieties (Sharon and Lis 1972) or some other category of agglutinins. Furthermore, the origin of these agglutinins remains undetermined.

Acton et al. (1969) also reported the occurrence of a serum hemagglutinin in *C. virginica.* Furthermore, they found that it is composed of identical subunits each of 20,000 daltons and contains fewer protein components than human immunoglobulins. These subunits are usually linked by non-covalent bonds and each subunit has a carbohydrate binding site. As this agglutinin can be inhibited by saccharides, it most probably is a true lectin.

Another serum agglutinin, which can be inhibited by bovine salivary gland glycoprotein, has been reported from *C. gigas* (Hardy et al. 1977b). It has been suggested that sialic acids serve as the binding site for this agglutinin. It remains undetermined as to whether the agglutinins reported by Acton et al. (1969) and Hardy et al. (1977b) originate within hemocytes.

Lysins

Although lysins have been reported from a few marine bivalves (Wittke and Renwrantz 1968; Anderson 1981), the only one that has been reported from *C. virginica* is lysozyme (MacDade and Tripp 1967a, b). This enzyme is synthesized in hemocytes and other cell types (Rodrick and Cheng 1974; Cheng and Rodrick 1975).

Parasite Immobilizing Factors

Two types of humoral parasite immobilizing factors (i.e., molecules that inhibit parasite mobility) are known in molluscs. The first is an apparently inducible serum factor reported from the gastropod *Biomphalaria glabrata* that immobilizes *Schistosoma mansoni* miracidia (Michaelson 1963, 1964; Lie et al. 1980). The second type is that reported by Cheng et al. (1966) from several marine bivalves. Specifically, they reported that a serum factor that seeps to the exterior of the bivalves *C. virginica, C. gigas, Tapes semidecussata, M. edulis, Modiolus demissus* (= *G. demissa*), *M. mercenaria,* and *M. arenaria* induces the encystment of the cercaria of *Himasthla quissetensis,* thus preventing it from penetrating and encysting *in vivo.* The source and nature of this serum factor remains unknown.

Cytotoxic Factor(s)

Although a cytotoxic factor(s) has been reported from the freshwater gastropod *Biomphalaria glabrata* infected with larval schistosomes (Bayne et al. 1980a, b), such a factor has not been reported from oysters. Moreover, there is some doubt as to whether such a separate factor actually exists in molluscs (Cheng and Combes 1990).

Lysosomal Hydrolases

By far, most of the studies directed at understanding molluscan humoral factors have concentrated on the occurrence and role of lysosomal hydrolases in internal defense. Recent reviews of this topic are available (Bayne 1983; Cheng 1983a, b, c; Chu 1988). The following represents a synopsis of what is known.

McDade and Tripp (1967a, b) were the first to report the presence of a lysosomal hydrolase, lysozyme, in the mucus and serum of *C. virginica.* Since then, as a result of finding lysosomes in granulocytes of different species of bivalves, including *C. virginica*

(Cheng 1975; Yoshino and Cheng 1976a), and their association with intracellular degradation (Cheng et al. 1974; Cheng and Cali 1975), identification of several lysosomal enzymes has been carried out. Cheng and Rodrick (1975) reported that β-glucuronidase, acid phosphatase, lipase, aminopeptidase, and lysozyme are associated with both the hemocytic and serum components of *C. virginica* hemolymph. Also, Cheng (1992a) reported the occurrence of α-mannosidase. The finding of these hydrolases in both cells and serum led to the investigation of the possible origin of the enzymes in serum. Cheng et al. (1975) demonstrated that when *M. mercenaria* granulocytes are actively phagocytosing bacteria, lysozyme is released from hemocytes into serum.

The kinetic properties of lysozyme from *C. virginica* hemolymph (both hemocytes and serum) were determined by Rodrick and Cheng (1974). The lytic activity of the oyster lysozyme on *Micrococcus lysodeikticus*, like that of egg-white lysozyme, is salt dependent, is relatively heat stable, and is very sensitive to changes in ionic concentration. The optimal pH of the oyster enzyme ranges from 5.0 to 5.5, depending on the buffer employed.

Yoshino and Cheng (1976b) studied alterations in the levels of aminopeptidase activity in the hemocytes and serum of *C. virginica* after *in vitro* exposure to heat-killed *Bacillus megaterium*. They reported that there is a significant elevation in intracellular aminopeptidase activity induced by the challenge; however, there is no increase in the activity of this enzyme in the serum. These data suggest that aminopeptidase is not released into serum by *C. virginica* hemocytes during phagocytosis although the bacterial challenge did stimulate hypersynthesis of this enzyme within hemocytes. Possibly the release of aminopeptidase from oyster hemocytes is only inducible by certain types of antigenic challenge and not by others. Whether this phenomenon holds true for some other lysosomal enzymes remains to be tested.

How are lysosomal enzymes released into serum? Foley and Cheng (1977), by studying *M. mercenaria* granulocytes, demonstrated that degranulation occurs during phagocytosis and this process represents the morphological basis for enzyme release from cytoplasmic granules. Subsequently, Mohandas et al. (1985) studied degranulation by employing scanning electron microscopy. They found that lysosomes migrate to the granulocyte surface and become extruded. The released lysosome is coated by two membranes: the inner is the native lysosomal membrane whereas the outer is comprised of a portion of the cell membrane of the granulocyte. As the lysosome is released into serum, rapid membrane fusion occurs at the site on the granulocyte surface where the lysosome was ejected (Fig. 15). Subsequently, the double membranous coat of each released lysosome is degraded in serum, possibly by previously released hydrolases. The double-layered nature of newly released lysosomes was confirmed by Mohandas and Cheng (1985) by transmission electron microscopy.

Hinsch and Hunte (1990) reported the extrusion of electron-dense particles from the cytoplasm of *C. virginica* hemocytes that were actively endocytosing latex beads by employing funnel-shaped pseudopodia. These "particles" may well have been lysosomes in the process of being released, as reported by Mohandas et al. (1985) and Mohandas and Cheng (1985). Hinsch and Hunte (1990) also reported the occurrence of primary phagosomes in which endocytosed beads are confined. Furthermore, the fusion of two or more primary phagosomes was also reported.

Further mechanistic details pertaining to the release of hydrolases of *C. virginica* hemocytes during phagocytosis were presented by Cheng (1992a, b), who reported that not all tested bacteria (*Escherichia coli*, Enteric Group 17, *Micrococcus roseus*, and *Klebsiella oxytoca*) induce the release of acid phosphatase and α-mannosidase from hemocytes into the suspension medium. Enzyme release is effected only by challenge with *E. coli* and Enteric Group 17 bacteria (Cheng 1992a). It was also demonstrated that a chelator, EDTA, is required for an ionophore (A23187)-stimulated release of acid phosphatase from *C. virginica* hemocytes. Furthermore, it was found that the ionophore-stimulated enzyme release is not Ca^{++}-dependent as in the case of vertebrate cells; however, there is a correlation between efflux of Zn^{++} with A23187-stimulated secretion of acid phosphatase (Cheng 1992b).

Lysosomal enzyme activity also has been studied from the ecological viewpoint. Specifically, Feng and

Canzonier (1970) reported that there are seasonal differences in serum lysozyme levels in *C. virginica* collected from the Navesink River, New Jersey. Generally, the enzyme activity is reduced in summer and does not deviate from the normal in oysters collected during winter. On the other hand, oysters with light to moderate infections of *Bucephalus* sp. exhibit significant elevations in serum lysozyme activity during both summer and winter, and the enzyme levels remain unchanged in oysters infected with both *H. nelsoni* and *Bucephalus* sp. Feng and Canzonier (1970) attributed the variations in lysozyme activity associated with the different infections to the pathogenicity of the parasites and the three-way interaction between the host, parasite(s) and ambient temperature. Chu and La Peyre (1989) reported that during a 1-year study in Virginia, serum lysozyme activity and total protein concentration exhibited seasonal fluctuations. Although there were variations between individual *C. virginica*, lysozyme levels were higher in winter than in summer and serum protein was higher during February and March. There was no linkage between serum lysozyme, protein concentration, and infection with *P. marinus*. Chu and La Peyre (1989) proposed that the observed seasonal changes in lysozyme and protein levels may be related to the reproductive cycle of the oyster.

Knowing that the overall pattern of enzyme activity during phagocytosis involves stimulation by certain antigens that results in the hypersynthesis of lysosomal enzymes and their subsequent release by the process of degranulation into serum, it remains to be determined whether the serum enzymes play a role in internal defense. Earlier, the antimicrobial property of lysozyme, which mediates the splitting of 1-4 linkages between N-acetylmuramic acid and N-acetylglu-

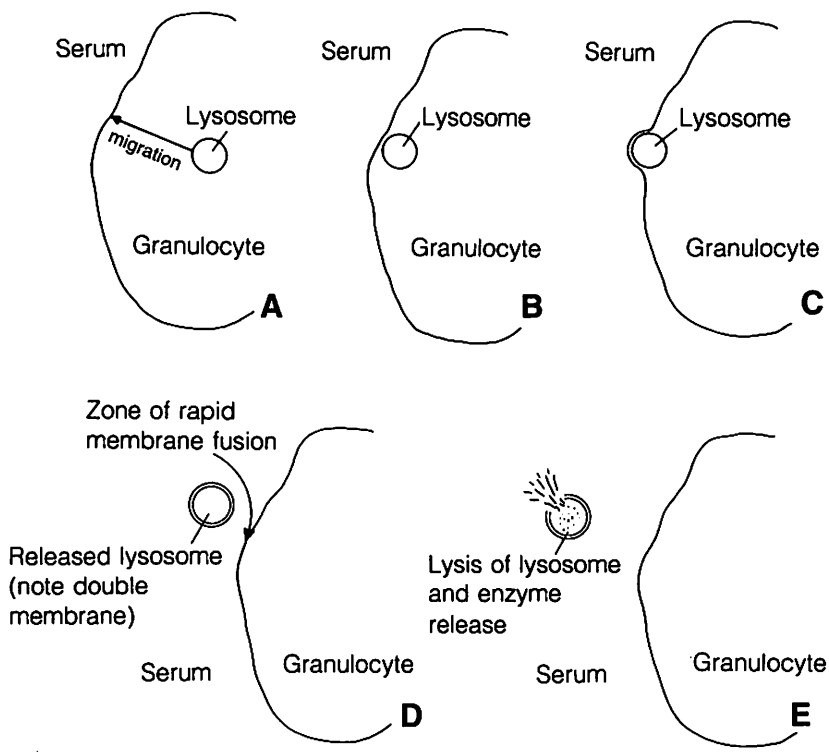

Figure 15. Schematic diagrams showing release mechanism of lysosomes from a bivalve granulocyte. (A) Lysosome migrating from endoplasm towards the plasma membrane. (B) Lysosome adjacent to the inner surface of the plasma membrane. (C) Lysosome protruding from the granulocyte. (D) Double-membraned lysosome exocytosed from the granulocyte. (E) Extracellular lysis of membranes surrounding the lysosome and release of enzymes. After Mohandas and Cheng (1985).

cosamine that occur in the cell walls of certain bacteria, was reported by McDade and Tripp (1967b). They found that this enzyme in the mantle mucus of *C. virginica* lyses certain Gram-positive bacteria, specifically, *Micrococcus lysodeikticus*, *Bacillus megaterium*, and *B. subtilis*. Subsequently, Rodrick and Cheng (1974) demonstrated that lysozyme in *C. virginica* hemolymph lyses *E. coli*, *Gaffkya tetragena*, *Salmonella pullorum*, and *Shigella sonnei*, in addition to *B. megaterium* and *B. subtilis*. It has no effect on *Staphyloccus aureus*. As a result of these studies and that of Cheng and Dougherty (1989), it appears that elevated levels of lysosomal enzymes in oyster (and other molluscan) serum can serve as defense molecules against susceptible microorganisms and metazoan parasites.

It is still not completely understood why certain invading organisms are destroyed and others are not. One postulation is the absence of substrates that are vulnerable to humoral response molecules, including lysosomal enzymes, of host origin on the surfaces of insusceptible invaders. A second possibility is that the release of lysosomal enzymes from their sites of synthesis, i.e., granulocytes and other cells (Yoshino and Cheng 1977) must be triggered by some component of the invader's somatic or metabolic antigens, or both. If such a triggering molecule(s) is absent, then enzymes would not be released at deleterious levels. A third possibility is that anti-enzymes are elaborated by the invader that inactivate the enzymes (Cheng 1977b).

In summary, a body of evidence exists that indicates there are inducible humoral protective molecules in oysters that are not immunoglobulins or opsonins. These are primarily lysosomal hydrolases that are restricted in their specificities and are released mostly from granulocytes. Lysosomal enzymes do occur at lower, ineffective concentrations in the serum of native oysters.

ACKNOWLEDGMENTS

This contribution is dedicated to the memory of S.Y. Feng (1929-1989) who was to have been the author. He was a scientific scholar of the highest order who contributed significantly to our understanding of molluscan hemocytes. The original information included in this contribution resulted from research supported by a grant (NA16FL0408-01) from the National Marine Fisheries Service, NOAA. Contribution No.5 from the Shellfish Research Institute.

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Chapter 9

Reproductive Processes and Early Development

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INTRODUCTION

The eastern oyster *Crassostrea virginica* is a gonochoric or dioecious alternate hermaphrodite (Coe 1943; Mackie 1984) with an annual reproductive cycle that culminates in spawning and external fertilization in the water column. The species is widely distributed on western Atlantic shores from northern temperate to sub-tropical environments that are characterized by wide seasonal environmental changes. At each location it reproduces during warmer periods when environmental conditions, including suitable planktonic food availability (see Langdon and Newell, Chapter 6) and high water temperatures, enable the larvae to grow and develop rapidly. Consequently, adult oysters are responsive to macroscale environmental changes that stimulate gametogenesis and spawning when conditions are suitable for larval survival. Gametogenesis is synchronized such that eggs and sperm are released concurrently, thereby ensuring fertilization and maximizing the numbers of zygotes. The final synchronization of spawning is effected by environmental cues in the surrounding water and the presence of gametes that stimulate or signal the onset of spawning in adjacent oysters. The resulting planktotrophic larvae develop in the water column.

This chapter describes essential aspects of the eastern oyster's reproductive adaptations, including the storage of nutrients necessary for reproduction and the initial stages of larval development. We do not present details on germ cell differentiation at the cellular level because this topic has been thoroughly reviewed for bivalve molluscs by Sastry (1979), but we describe briefly the early stages of development (see Elston 1980 for more details). Details of the structure of the gonad are given by Eble in Chapter 2 and information on larval behavior and metamorphosis is summarized by Kennedy in Chapter 10. Other relevant reviews of reproduction are by Andrews (1979) for oysters, by Barber and Blake (1991) for scallops, by Seed and Suchanek (1992) for mussels, and by Bayne (1976), Sastry (1979), and Mackie (1984) for bivalves in general.

SEX RATIOS AND CHANGES WITH SIZE

Crassostrea virginica is a protandric species, i.e., when individuals first mature they generally function as males, although even in their first year larger oysters are more likely to be female than are smaller oysters (Needler 1932a; Coe 1934). Andrews (1979) reported that in the James River, Virginia, 90% of oysters smaller than 35 mm shell height, and as young as 6 weeks post-settlement, functioned as males in the season in which they settled. Sex reversal usually occurs when the gonad is undifferentiated between spawning seasons. As individuals grow, the proportion of functional females in each size class increases, with an excess of females occurring among larger (and presumably older) animals (Galtsoff 1964). Hermaphrodites (individuals containing both eggs and sperm) are rare in most species of oysters; in eastern oysters they form less than 0.5% of populations exam-

ined (Burkenroad 1931; Needler 1932a; Coe 1934; Berg 1969; Kennedy 1983).

Although eastern oysters normally switch from functioning as males to functioning as females, there is evidence that the process is reversible in subsequent years (Table 1). The percentage of individuals that changed sex has varied widely from study to study and from year to year, with an overall average of 16% of the males and 29% of the females changing sex. In Galtsoff's (1964) five-year study, 31 of the 68 survivors at the end of the fourth breeding season had changed sex at least once over the experimental period, with 18 changing only once, ten twice, two three times, and one four times. Because the eastern oyster exhibits sex reversal, its sex is presumably not determined by a sex chromosome. Coe (1943) suggested that certain genes may stimulate or inhibit developmental agents that lead to an individual becoming male or female.

The adaptive significance of sex reversals in oysters in general, and the factors influencing them, are not yet clear. Egami (1953) tested the hypothesis first proposed by Amemiya (1935) that food limitation leads to a greater preponderance of male oysters in a population. His experiment involved excising portions of gill tissue in some Pacific oysters, *Crassostrea gigas*, and removing portions of mantle tissue in others, both in October and in January. When the oysters were examined the following summer, in the October treatment there were significantly more males in the gill-excised group than in the mantle-excised group or in the controls (no tissue excised), but there was no significant difference in sex ratio between mantle-excised and control oysters. In contrast, there were no significant differences between any treatment or control groups in the January treatment. These results support the hypothesis that reduced food intake reduces the ratio of female to male oys-

Table 1. Sex change (percentage) between breeding seasons for mixed groups of individual oysters of known sex. N = sample size.

Year	Change to opposite sex				Reference
	Males (%)	N	Females (%)	N	
—	58	138	23	234	Amemiya 1929[a]
—	21	24	42	12	Needler 1932b
—	8	125	13	61	Galtsoff 1937
—	26	27	25	16	Burkenroad 1937
1933	19	31	62	26	Needler 1942[b]
1934	7	41	25	16	Needler 1942[b]
1935	19	42	53	15	Needler 1942[b]
1936	31	42	47	15	Needler 1942[b]
1	8	119	17	63	Galtsoff 1964[c]
2	11	88	24	38	Galtsoff 1964[c]
3	23	65	9	33	Galtsoff 1964[c]
4	12	25	25	24	Galtsoff 1964[c]
5	7	15	6	18	Galtsoff 1964[c]

[a] *Crassostrea gigas* (all others in this table are *C. virginica*).

[b] Same group of oysters was followed from 1932 to 1936.

[c] Same group of oysters was followed for a 5-year period (dates not given).

ters in a non-reproducing population. However, once gametogenesis has been initiated, *C. gigas* loses the ability to change its sex for that season.

Nutritive stress also appears to influence sex determination in eastern oysters. Bahr and Hillman (1967) and Davis and Hillman (1971) stressed *C. virginica* by starving them and filing the edge of the growing shell. Both studies reported that lack of food, together with the need for energy to repair damaged shell, shifted the sex ratio towards an excess of males compared with control oysters that were fed and had no shell damage.

These observations suggest that poor environmental conditions may result in a change in the sex ratio, with an excess of males over females. This view is supported by the work of Tranter (1958), who reviewed the literature on sex determination in hermaphroditic bivalves and concluded that germ-cell rudiments differentiate towards maleness when stored food reserves are low and towards femaleness when reserves are high. Furthermore, Sastry (1968) observed that oocyte growth in hermaphroditic bay scallops, *Argopecten irradians concentricus,* is suppressed in the absence of food, with only spermatozoa developing under low food and temperature conditions. According to some authorities (e.g., Russell-Hunter 1979), sperm are energetically "cheaper" to produce than eggs, which have substantial yolk reserves, although Vahl and Sundet (1985) suggest that there may be no net energy savings associated with sperm production. The adaptive significance of altered sex ratios in eastern oysters during periods of stress needs to be more carefully characterized.

Cox and Mann (1992) reported a significantly greater number of male than female eastern oysters from four locations in the James River, Virginia. Conversely, previous data from one of these locations had demonstrated a sex ratio of approximately unity for oysters larger than 60 mm shell height (Morales-Alamo and Mann 1989). Cox and Mann (1992) postulated two hypotheses to explain this change in sex ratios between the two studies. The first was that smaller oysters were analyzed in their own samples, which therefore contained a larger proportion of males. The second was that more oysters may have spawned as males owing to a stress associated with infections by the protozoan parasites *Haplosporidium nelsoni* (MSX disease) and *Perkinsus marinus* (Dermo disease). Although these parasites are inactive during the winter period when sex is determined, they reduce the amount of nutrients stored during the preceding summer. Thus, parasitic stress may interfere with reproductive processes in the following season (see Effects of Environmental Stress Factors section, p. 350). Conversely, when Ford et al. (1990) examined the effects of *H. nelsoni* on sex ratios of *C. virginica* they found a three-fold increase in the ratio of females to males in infected oysters. Because there was no evidence of sex-associated differential infestation or mortality rates, the authors concluded that the parasite probably inhibits spermatogenesis rather than oogenesis. Further research is needed to determine the effects of stress, including poor nutrition and the timing of the stress in the reproductive cycle, on sex determination in *C. virginica.*

There is evidence that determination of sex in an individual eastern oyster may be influenced both by the sex of nearby oysters and by their proximity. Needler (1932a) found that male eastern oysters from Prince Edward Island, Canada, tended not to change sex when close to females. Burkenroad (1931) reported that the ratio of females to males in large (>4 cm shell height) eastern oysters growing unattached in Louisiana was 3.9:1, which differed from the population ratio of 1:1. For oysters in clumps the female to male ratio was 1.7:1 if their valve margins were more than 4 cm apart, and 1:1 if less than 4 cm apart, the difference being statistically significant. In South Carolina, Smith (1949) noted significantly different female to male ratios of 1.1:1 for eastern oysters growing singly versus 0.6:1 for aggregated adults. In Louisiana, Menzel (1951) found a female to male ratio of 1.2:1 in 4 to 9 cm eastern oysters living on dead shell, a significantly higher value than the 0.3:1 observed for 5 to 7.5 cm individuals attached to older (>2 years) adults.

Kennedy (1983) examined the influence of proximity in determining the sex of adult *C. virginica* held over winter in trays containing either all males (N=66), all females (N=92), or an equal number of both sexes (N=34 each). By the following July, 31% of oysters in the tray of males had changed to female and 33% of those in the tray of females were male.

The sex ratio in the mixed tray remained 1:1, although there was evidence that two of ten oysters that were previously female had changed to male. Before initiating a field study, Kennedy (1983) hypothesized that because recruitment had been below average for a period of 10 years in central Chesapeake Bay, the remaining older eastern oysters would be predominantly female. However, for oysters measuring 4 to 19.5 cm shell height, in only 11 of 29 samples from 23 oyster beds did the ratio of females to males differ significantly from 1:1, and in only 3 samples did the ratio exceed 2:1, suggesting that natural populations of adult eastern oysters maintain a relatively balanced sex ratio. The results of these various studies show that the presence of one sex influences the abundance of the other. It is likely that one or more pheromones may be involved in sex determination, but the nature of the compound remains to be determined.

FACTORS INFLUENCING GAMETOGENESIS

The gametogenic cycle of the eastern oyster (Kennedy and Battle 1964), in common with that of many other marine invertebrates from temperate and boreal environments, involves the storage of energy reserves to support gametogenesis, the production and accumulation of gametes by cell proliferation and differentiation, the release of ripe gametes, and a recovery or resting period (Giese and Pearse 1974; Giese and Kanatani 1987). The germinal epithelium remains undifferentiated during winter, the precise duration depending upon geographic location and local water temperatures. At this time, metabolic activities at the cellular level undoubtedly continue, but neither follicular development nor gametogenesis is evident in histological sections. As temperatures rise in spring, the germinal epithelium proliferates, gamete development and growth begin, and follicles enlarge and ripen. Maximum follicular proliferation, vitellogenesis, and production of mature gametes occur just before spawning. When spawning is complete (the spent stage), follicles shrink and are invaded by amoebocytes that resorb any unspawned gametes (see Cheng, Chapter 8), and the superficially quiescent (resting) state resumes.

The spawning period in the eastern oyster varies with geographic location (Table 2), with gametes ripening earliest at the southern end of the species' distribution. Although at the population level small numbers of gametes are probably continuously released throughout the spawning season, there are generally one or more major peaks of spawning activity (Table 2). For example, on the Gulf coast, and north up the Atlantic coast to Virginia, major spawning occurs in the spring, with minor spawning through the summer followed by another major spawning in the fall (Hayes and Menzel 1981). In contrast, in populations near the northern distributional limit, the major spawning event is restricted to the summer, with the degree of subsequent gamete development being variable between years.

Reproductive events are coordinated by endogenous factors, such as stored nutrients and neuroendocrine compounds, and by exogenous cues or stimulants such as salinity, temperature, and pheromones (Giese 1959; Giese and Pearse 1974; Sastry 1975). Although it is convenient to categorize factors in this way (Fig. 1), it is misleading to consider them as separate effectors, because they interact. For example, an exogenous factor such as temperature can directly affect metabolic processes associated with gametogenesis, but may also act indirectly by controlling feeding rate and hence the rate of nutrient acquisition. These interactions are clearly demonstrated by aquaculture techniques in which eastern oysters (Dupuy et al. 1977) and Pacific oysters (Robinson 1992) can be brought into spawning condition by use of appropriate food and temperature conditions.

Endogenous and Exogenous Control of Reproduction

Information on the occurrence and function of hormones in bivalves is extremely limited (reviewed by Joosse and Geraerts 1983). Most of the available data for the neuroendocrine regulation of gametogenesis and nutrient storage have been obtained for the blue mussel, *Mytilus edulis*, and comprehensively reviewed by De Zwaan and Mathieu (1992). This re-

search suggests that there is a complex feedback between nutrient reserves and the initiation of gametogenesis and vitellogenesis (Gabbott 1975, 1983; Sastry 1979). Further research is required to determine the role of neurosecretions and the mobilization of nutrient reserves in regulating gametogenesis in the eastern oyster.

Our understanding of the influences of exogenous factors on invertebrate reproduction is also limited (Giese and Pearse 1974; Sastry 1979; Giese and Kanatani 1987). Considerable attention has been devoted to understanding the role of water temperature, a cyclically varying environmental factor that serves as a conservative seasonal signal. Thorson (1946) referred to the relationship between invertebrate reproduction and water temperature as "Orton's Rule," and noted that gradual temperature changes may influence gametogenesis whereas sharp increases induce spawning. Temperature is an important factor controlling reproduction but its effects are complex (see Shumway, Chapter 13).

There are few data on the influence of environmental factors on gametogenesis in *C. virginica*, but there is considerable information for the bay scallop *A. i. concentricus* (for reviews see Sastry 1979; Barber and Blake 1991). These studies suggest that temperature and food supply are important environmental factors regulating reproduction in bay scallops. In North Carolina, gonadal growth of *A. i. concentricus* coincides with the annual peak of phytoplankton production (Sastry 1966), a reflection of the importance of food conditions in the timing of the reproductive cycle. In the early phase of the gametogenic cycle and in the post-spawning resting stage, bay scallops require an adequate food supply as well as a suitable temperature to stimulate gonad growth. Under poor food conditions, tissue reserves are used for maintenance metabolism rather than for gametogenesis. Food supply appears to be less critical after certain minimum reserves have accumulated in the gonad, and gonad maturation then occurs at a rate that is dependent on temperature. However, even if the mini-

Table 2. Spawning periodicity of the eastern oyster throughout its natural range and in Hawaii. After Kennedy (1986).

Location	Study period	Timing of spawning	Authority
Prince Edward Island, Canada	1961 to 62	Jun to Aug	Kennedy and Battle (1964)
Long Island Sound, Connecticut	1937 to 56	Start: Jun 6 to Jul 3. End (90%): Aug 15 to Sep 6	Loosanoff (1965)
Chesapeake Bay: Maryland	Not stated	Early Jun to early Oct; usually peaks in July	Chesapeake Biological Laboratory (1953)
	1977 to 78	Late May to Sep	Kennedy and Krantz (1982)
Chesapeake Bay: Virginia	Many years	Late Jun to late Sep	Andrews (1979)
Gulf of Mexico, Florida	1949 to 50	Late Mar to late Oct, varying with location; multiple spawnings	Ingle (1951)
	Not stated	May to Oct	Butler (1965)
	1978	Late Apr to early Oct	Hayes and Menzel (1981)
Hawaii	1963 to 65	Feb to Nov; mainly Mar to Oct	Sakuda (1966)

mal reserves have accumulated, the gonad does not develop as extensively in the continued absence of food as it does in the presence of adequate food. Low temperatures can be inhibitory in that well-fed, reproductively quiescent bay scallops held at 15°C pass through the early gametogenic stages, but oocytes do not enter the normal growth phase (Sastry 1968). At 20°C, however, bay scallops complete normal gonad development when they are well-fed but not when they are starved. Thus normal reproductive development requires both a certain minimum temperature and an adequate food supply.

Barber and Blake (1983) studied reproduction of the bay scallop in Florida, the southern distributional limit of the species. Gametogenesis in this southern population was initiated at a higher temperature and later in the year than in northern populations. The authors postulated that oocyte growth in this species responds to food supply and a population-specific minimum temperature that varies over the species' geographical range. No similar studies have been undertaken on the eastern oyster, although it is likely that it too depends both on suitable food supplies and temperatures for the initiation of gametogenesis. The fact that southern populations of *C. virginica* initiate gametogenesis and spawn earlier in the year than northern ones (Table 2) suggests that water temperature may be the most dominant factor. Research into the direct effects of temperature on the metabolism and rate of gametogenesis of *C. virginica* has been reviewed by Shumway in Chapter 13.

From literature reports on the effects of temperature on spawning of oysters from Texas, New Jersey, and Long Island Sound, Stauber (1950) concluded

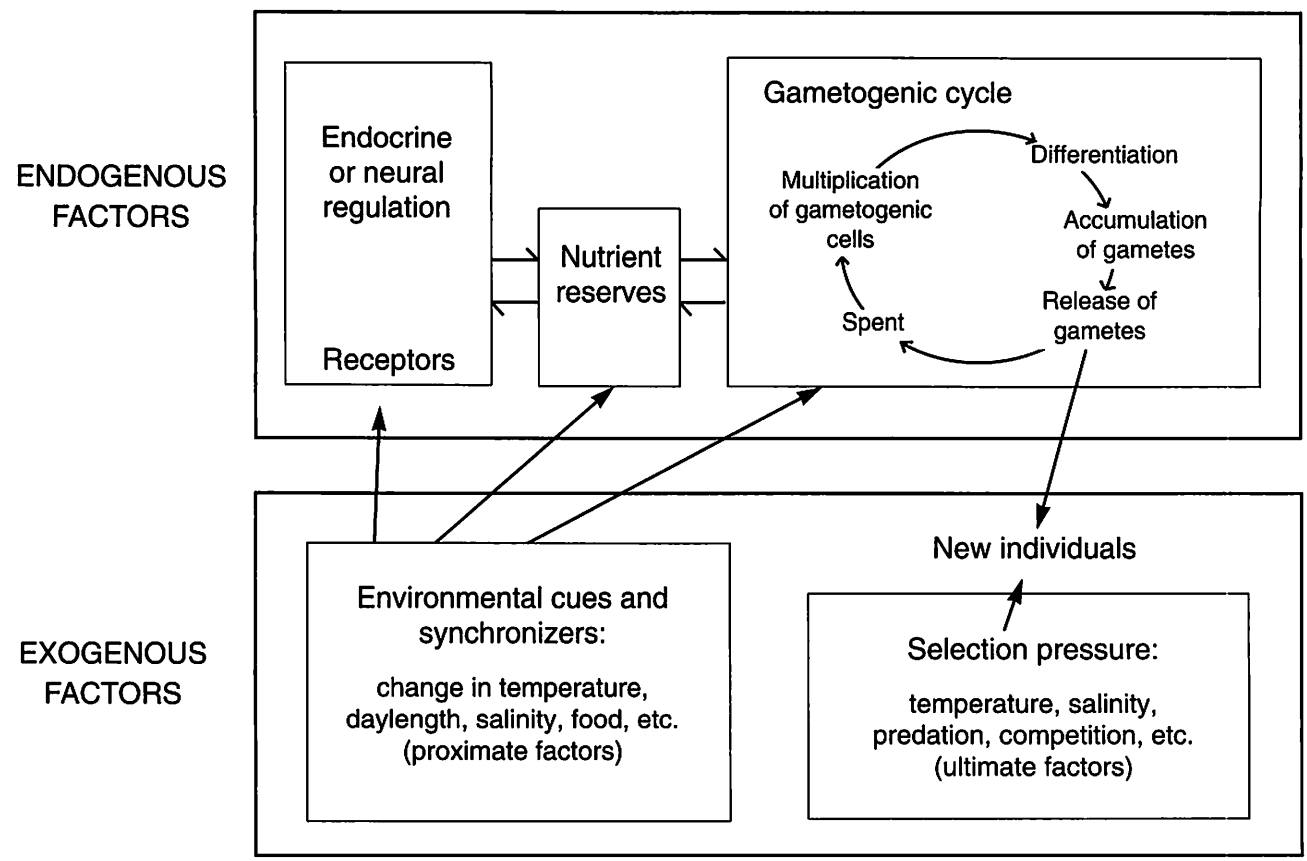

Figure 1. Schematic representation of exogenous and endogenous factors thought to be important in controlling the reproductive process in oysters. Based on a diagram in Giese and Pearse (1974).

that there are probably at least three physiological races of *C. virginica* along the eastern seaboard of North America. Loosanoff and Nomejko (1951) and Loosanoff (1969) undertook transplantation experiments to determine the relative contributions of genotypic and phenotypic responses to changes in water temperature in terms of the timing of gametogenesis and spawning. Because some stocks transplanted to the common environment of Long Island Sound maintained differences in the time of initiation of gametogenesis and spawning, Loosanoff and Nomejko (1951) and Loosanoff (1969) concluded that these processes in eastern oysters were under genetic control to some extent. A long-term transplantation study of eastern oysters into Delaware Bay from Long Island Sound was carried out by Barber et al. (1991) to study this problem further. The authors found that Long Island Sound oysters inbred and reared within Delaware Bay for six generations initiated gonadal development and started spawning about one month earlier than eastern oysters native to Delaware Bay that had been maintained under identical conditions, i.e., the Long Island Sound oysters retained their original reproductive cycle. These results indicate a strong genetic component to the reproductive cycle that enables eastern oysters to complete reproduction and development over their entire distributional range, despite different water temperatures prevailing at various locations.

Barber et al. (1991), however, could find no evidence from allozyme or mtDNA genetic studies reported in the literature that these various eastern oyster populations were in fact genetically differentiated. This does not prove that they are not different, because there appears to be no reliable procedure for positively delineating genetic boundaries between oyster populations (see Gaffney, Chapter 11). In this respect *C. virginica* populations are similar to populations of *Mytilus edulis* studied by Thompson and Newell (1985) which exhibit genetic adaptations to prevailing ambient water temperatures even though allozyme studies suggest the populations do not differ genetically.

The relative contributions of genotypic and phenotypic adaptations to the reproductive cycle exhibited by different species of bivalve molluscs have also not been fully resolved. In *M. edulis*, differences in the gametogenic cycle have been linked to individual genotypes (Hilbish and Zimmerman 1988). In genotypes with low rates of nitrogen accumulation, the peak reproductive condition was delayed by six weeks compared with genotypes with higher rates of nitrogen accumulation. In transplantation studies with *M. edulis* (Widdows et al. 1984) and the clam, *Scrobicularia plana* (Worrall and Widdows 1983), physiological rate functions acclimatized within 3 months and attained rates similar to those of individuals from the adjacent local populations. For both species, however, acclimatization was not perfect. Because blue mussels and clams were not studied for longer than 6 and 3 months, respectively, it was not ascertained if acclimatization would ever be complete or if the residual differences were genetically based. Widdows et al. (1984) did report that both in the blue mussels that they transplanted and in those from other transplantation studies, differences in the number and seasonal cycle of nutritive storage cells were maintained between populations following transplantation. Because the reproductive cycle of bivalves is so dependent on processes that occur over extended periods of time, such as nutrient storage and development of germinal tissue, it is likely that complete acclimatization of the reproductive cycle may take at least a complete annual cycle.

Other studies have shown that site-specific variations in the gametogenic cycle of blue mussels (Newell et al. 1982) and various species of scallop (Bricelj et al. 1987; MacDonald and Thompson 1988) can be associated with phenotypic adaptations to local seasonal patterns of food availability. Such a capacity for phenotypic response to prevailing conditions seems likely to allow a species with widely dispersing planktotrophic larvae to maximize reproductive output. Conversely, fixed times of reproduction exhibited by spatially separated populations of eastern oysters appears to be maladaptive. The evolution of such fixed reproductive patterns requires that there must be little mixing of oyster larvae from stocks genetically adapted to conditions in different environments. This is in spite of the fact that only a limited exchange of individuals between populations is necessary to ensure homogeneity of the species over large

geographic areas (see Gaffney, Chapter 11). Furthermore, the maintenance of these genetically determined responses in a commercial species such as the eastern oyster is even more surprising considering the extensive commercial transplantation that has taken place within their geographic range over the last century (see Carlton and Mann, Chapter 20). It is possible, however, that high post-settlement mortality in individuals with a maladapted genotype will ensure that genetic differences are maintained. Further research into the relative importance of genetic and phenotypic reproductive adaptations to prevailing environmental conditions in bivalve molluscs, including eastern oysters, will be required to answer these questions definitively.

BIOCHEMICAL ENERGY STORAGE CYCLES

Eastern oysters, in common with many other species of temperate shallow-water marine bivalves (Giese and Pearse 1974; Bayne 1976), exhibit a pronounced seasonal cycle in the synthesis, storage, and use of biochemical energy reserves. Typically, reserves are sequestered during periods of high food availability in late summer and fall, at which time the major energy requirements for somatic and germinal growth have already been satisfied. The reserves are then subsequently used to initiate gametogenesis and to maintain metabolism during periods of low food supply and reduced feeding in the winter. The most important storage compound in oysters is glycogen, which serves as a substrate to fuel gametogenesis. Glycogen can also be used to synthesize lipids that are transferred to the yolk of developing oocytes during vitellogenesis. In this way oysters partially uncouple the processes of energy intake and gamete production, allowing gametogenesis to begin during the winter when food intake is at a minimum.

The roles of various tissues in the storage of energy reserves in oysters have not been examined as critically as in some other bivalves (Gabbott 1976, 1983; Thompson and MacDonald 1990). Histological examination of *C. virginica* has revealed, however, that glycogen is stored in the interstitial tissue of the gonad, as well as in the digestive gland and the "Leydig cells" that are concentrated between the digestive gland and the gut wall (Cheng and Burton 1966). The large adductor muscle found in scallops serves as an important site for glycogen storage (Barber and Blake 1991), but this function is unlikely in eastern oysters because the adductor muscle comprises a relatively small proportion of the body mass.

Although the literature on the proximate biochemical composition of *C. virginica* is extensive, much of it is difficult to interpret or to relate to other studies. There are a number of reasons for this difficulty, including inadequate sample replication, which precludes statistical analysis. The literature spans many decades, and an unusually wide variety of analytical techniques has been employed. In many instances, the sum of the lipid, carbohydrate, protein, and ash concentrations expressed as percentages of dry weight lies far from the theoretical value of approximately 94% (allowing for bound water, Gnaiger and Bitterlich 1984). This discrepancy is presumably attributable to poor technique. Carbohydrate is sometimes obtained by difference after the other components have been determined, which can mask any errors in the measurement of one or more components. Very often, data are expressed in terms of wet tissue weight, which confounds the analysis of seasonal data because water content varies according to nutritive and reproductive condition.

Most of the published data are for proximate analyses of the whole body (less shell). Very rarely have there been separate analyses of tissues such as the digestive gland and gonad, in part because such analyses are difficult to accomplish in all species of oysters, and also because the primary interest in most studies of commercially important species is the entire meat. In many cases, authors have failed to grasp the importance of measuring the weight of the tissue and the shell size of the oyster they analyze as well as the concentration of the component of interest. These data are required for the calculation of the content of a specific biochemical component, i.e., the total amount of the component present per oyster of standard shell size, and for the estimation of quantities such as the net energy loss during gametogenesis. Expression of biochemical constituents solely as percentages of tissue weight can be misleading because any

change in one component is necessarily reflected in the others (Barnes et al. 1963; Gabbott 1976). Furthermore, the total amount of a given constituent per oyster may increase or decrease significantly without a commensurate change in its concentration in the tissue if other components are changing proportionally. The failure of many published studies to meet these statistical, analytical, and logical criteria has severely restricted our attempts to make comparisons among data sets, which is unfortunate because it means that information on oysters cannot be optimally utilized. In contrast, the relationships among energy storage cycles, gametogenesis, and environmental factors are more completely understood in the blue mussel (Gabbott 1976, 1983) and in several species of scallops (Thompson and MacDonald 1990).

In this section we consider the way in which oysters synthesize and store biochemical energy reserves in various tissues. We discuss the relationships between these processes and those of gametogenesis and spawning, and how the seasonal nutrient storage cycle may be modulated by changes in environmental conditions, particularly nutrition. While the primary focus is on the eastern oyster and the closely related Pacific oyster, data from other ostreids will be used where necessary for comparative purposes or to illustrate a point for which information is inadequate or unavailable for *Crassostrea* spp.

Glycogen

It is well known that there is a relationship between the proximate biochemical composition of oysters (*Crassostrea* and *Ostrea* spp.) and the gametogenic cycle, and that glycogen plays a particularly important role (Masumoto et al. 1934; Chipman 1948; Engle 1951; Walne 1970; Holland and Hannant 1973; Walne and Mann 1975; Mann 1979; Sidwell et al. 1979; Perdue et al. 1981; Deslous-Paoli et al. 1982a,b; Whyte and Englar 1982; Perdue and Erickson 1984; Allen and Downing 1986; Muniz et al. 1986; Deslous-Paoli and Héral 1988). According to Chipman (1948) and Engle (1951), glycogen concentrations in *C. virginica* from a variety of localities are minimal immediately after spawning in August, though they increase during the fall and early winter (a process commonly called fattening). Both authors report that maximum values are reached in March before decreasing during gametogenesis from April to July. Sidwell et al. (1979), however, found that maximum glycogen values occurred during May in eastern oysters from Maryland, Alabama, and Louisiana. Such inter-population differences are presumably attributable to local variations in factors influencing the timing of the gametogenic cycle, which may also vary between years. Furthermore, there is some evidence that whereas individuals from southern populations (e.g., Louisiana, Alabama) may continue to feed and synthesize glycogen throughout the winter (Sidwell et al. 1979), those living further north become inactive when temperatures fall below 8°C (Newell, unpublished data), so that glycogen synthesis is interrupted during the colder winter months. Some support for this interpretation was provided by Ruddy et al. (1975), who observed that eastern oysters held at 14 to 19°C were able to exploit a large phytoplankton bloom and synthesize glycogen, whereas a control group at 2 to 7°C were unable to do so, presumably because they ceased feeding at such low temperatures. There can also be a net loss of glycogen during the winter, when it may be catabolized to meet maintenance requirements as a result of poor food conditions, e.g., in *C. gigas* grown at high densities in western France (Deslous-Paoli and Héral 1988).

A comprehensive study of nutrient storage cycles in *C. virginica* was undertaken by Barber et al. (1988a, b) as part of an investigation into the biochemical consequences of infestations by *Haplosporidium nelsoni* in Delaware Bay. In this study, glycogen, lipid, and protein content of the tissues were expressed both as percent content and the total amount of each constituent present in an oyster of 10 cm shell height. These authors also reported seasonal changes in reproductive condition, quantified histologically as the percentage of the visceral mass that contained germinal tissue. For our discussion we have only used data that they obtained from oysters uninfected by MSX (Fig. 2). Glycogen content (mg oyster^{-1}) was at a minimum at the time of peak reproductive condition (Fig. 2) in May through June. The oysters spawned between mid-June and early July, after which the glycogen

content increased to a maximum in August, followed by a decline through the winter months.

Studies of other *Crassostrea* spp. confirm this basic pattern but exemplify annual and site-specific variations. For example, Masumoto et al. (1934) reported that glycogen concentrations in *C. gigas* from Kusatsu, Japan, are lowest in September when spawning is complete, and increase throughout the winter to attain a peak in March before spawning begins and glycogen concentrations decrease (Fig. 3A). In *C. gigas* grown in non-tidal ponds near the Bay of Marennes-Oléron on the Atlantic coast of France, glycogen concentration and content reach minimum values in July, immediately before spawning when the gamete complement is greatest (Deslous-Paoli et al. 1982b). After spawning takes place in August, glycogen concentration and content increase until November, then decrease throughout the winter, although the main use of glycogen occurs during the major gametogenic phase in June and July. In the Bay itself, however, glycogen synthesis and depletion are less predictable and vary from year to year (Deslous-Paoli and Héral 1988). Thus, in some years glycogen concentration increases in the period immediately preceding spawning (August) and decreases thereafter, whereas in other years the pattern is similar to that seen in C. *virginica* on the east coast of North America, i.e., an increase in glycogen concentration during late fall, followed by a decrease during the later phases of gametogenesis. In the first of the three years for which data are presented by Deslous-Paoli and Héral (1988) for *C. gigas* (Fig. 4A), there was clear evidence of the use of glycogen reserves during the winter, which the authors attributed to unfavorable food and temperature conditions, although there was no strong evidence for this. In the second year, however, glycogen concentrations increased rather than decreased between November and April. Although in the third year there was some evidence for a decrease in glycogen during winter, sampling was infrequent and the data are equivocal. The increases in glycogen concentrations usually observed in March and April are probably associated with the spring phytoplankton bloom, as seen in the chlorophyll data (Deslous-Paoli and Héral 1988), but there is insufficient information to go further in relating glycogen concentration or content to food availability.

Lipid and Protein

Seasonal changes in lipid values reported in the oyster literature are generally more difficult to interpret than those for glycogen. In a study of *C. virginica* from Delaware Bay the percent lipid content remained between 10 and 15% throughout the year (Fig 2; Barber et al. 1988b). Surprisingly, the absolute amount of lipid was highest in August, a time when the oysters were in a quiescent reproductive condition. Protein concentrations followed a pattern similar to lipid, with a fairly uniform percent composition throughout the year but with the highest amount per oyster in August (Fig. 2).

The pattern of lipid accumulation appears a little clearer in *C. gigas*, where Masumoto et al. (1934) recorded lipid values of 9 to 14% dry weight in females from Kusatsu, Japan, during the fattening phase from October to March (Fig. 3B). Lipid increased to 18% dry weight during gametogenesis (April to June), then fell rapidly to 8% dry weight after spawning was complete in August, presumably as a result of the release of lipid-rich eggs. Deslous-Paoli et al. (1982a, b) and Deslous-Paoli and Héral (1988) also observed an increase in lipid during the gametogenic phase in *C. gigas* cultured in western France, with values in females reaching 2 to 15% dry weight, followed by a decrease during spawning (Fig. 4B). In contrast, Whyte and Englar (1982) reported lipid values of 7 to 8% dry weight throughout the year in *C. gigas* grown in British Columbia, Canada.

Trider and Castell (1980) found that the concentration of polar lipids showed no seasonal variation in *C. virginica* from Prince Edward Island, Canada, but neutral lipids decreased considerably during spawning in July. Polar lipids, especially phospholipids, function mainly as structural components of membranes, whereas neutral lipids are accumulated as an energy reserve. Thus the loss in the gametes released would be relatively greater for the latter than the former, at least in females. Trider and Castell (1980) also determined the fatty acid composition of the neutral lipid fraction. The amounts of saturated and monounsaturated fatty acids relative to total fatty acids were greatest after spawning in July, but the polyunsaturated fatty acid fraction (PUFA) was lowest. In particular,

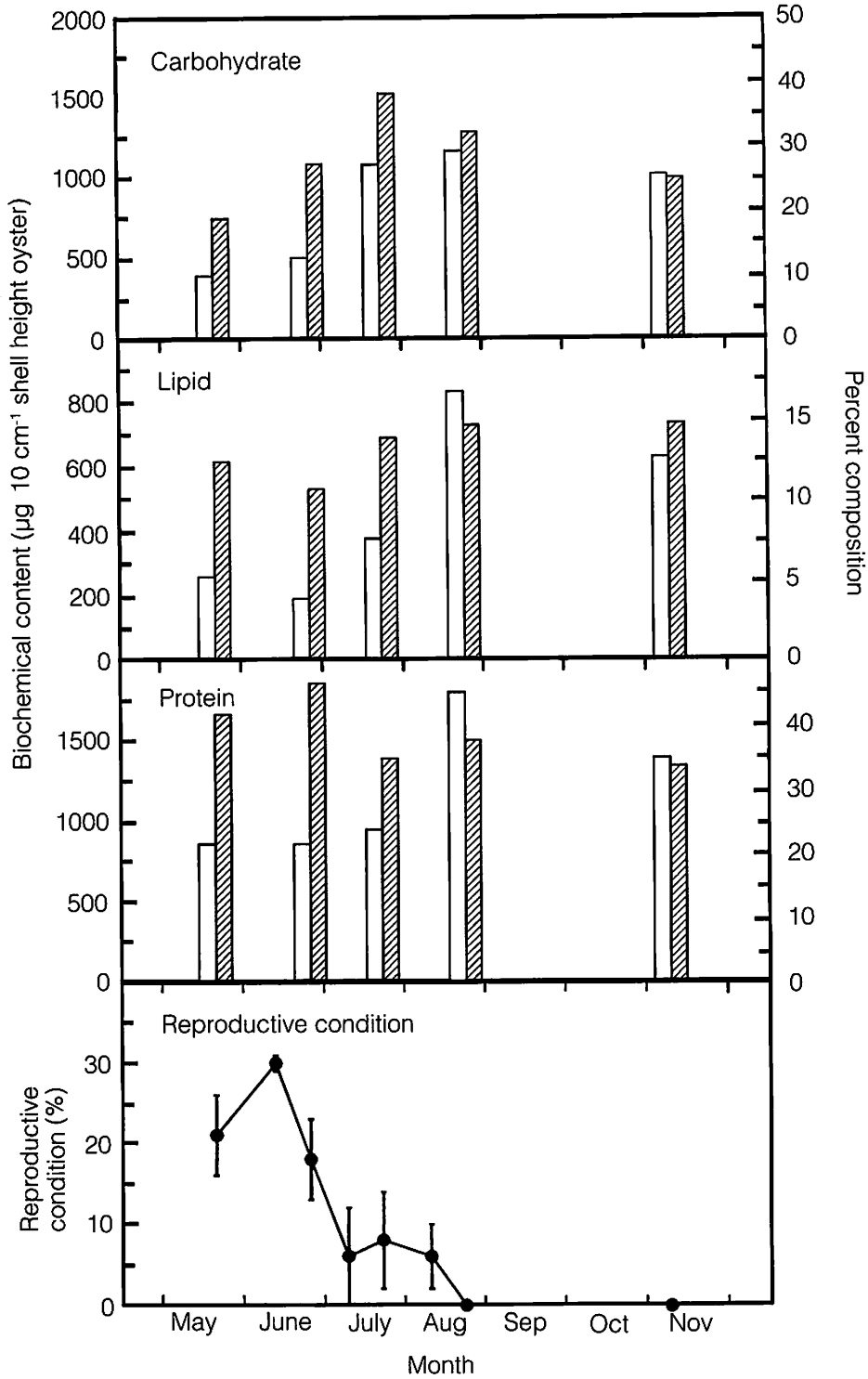

Figure 2. Seasonal variation in the proximate composition of tissue and in reproductive condition for *Crassostrea virginica* in Delaware Bay. Data are reported as total content for an oyster of 10 cm shell height (open bars) and as percent composition (hatched bars) for total carbohydrate, lipid, and protein. Reproductive condition was calculated as the area of germinal tissue expressed as a percentage of the area of the entire visceral mass in histological sections. Data are from Barber et al. (1988a, b) and are means (± SD for reproductive condition data) of between 3 and 21 disease-free oysters. Data for the September sampling time are not presented because only one uninfected oyster was analyzed at that sampling time.

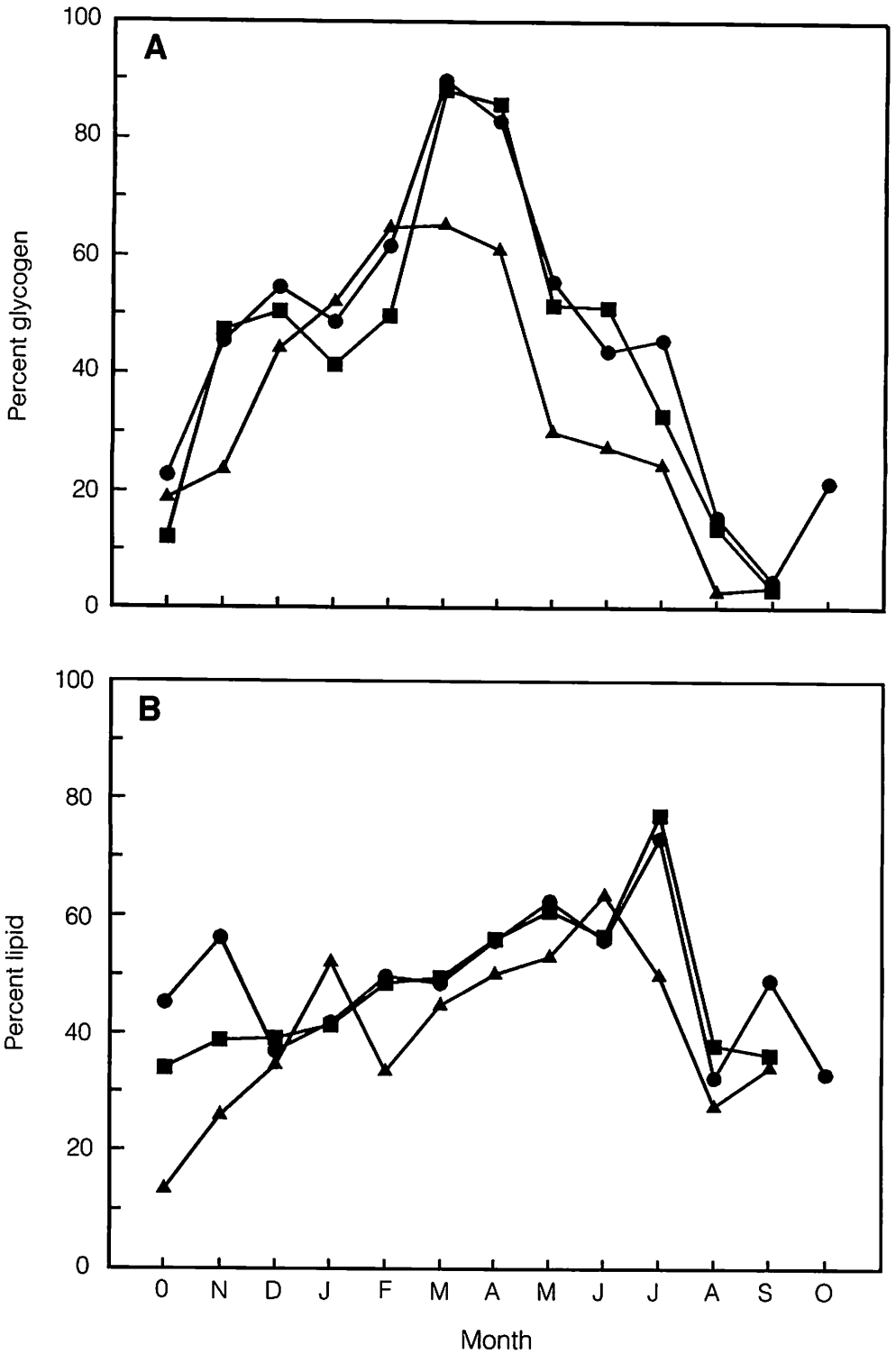

Figure 3. Seasonal cycle of glycogen (A) and lipid (B) concentration (expressed as percent of dry weight) in female *Crassostrea gigas* at Kusatsu, Japan, a site of rapid oyster growth and at Zigozen, Japan, where oyster growth is slower. Three groups of oysters were monitored; Kusatsu oysters held at Kusatsu (●); Kusatsu oysters transplanted to Zigozen (▲); and Zigozen oysters transplanted to Kusatsu (■). Data from Masumoto et al. 1934. (Note that only percent composition could be plotted because data necessary to convert values to total content for an oyster of given shell size were not provided.)

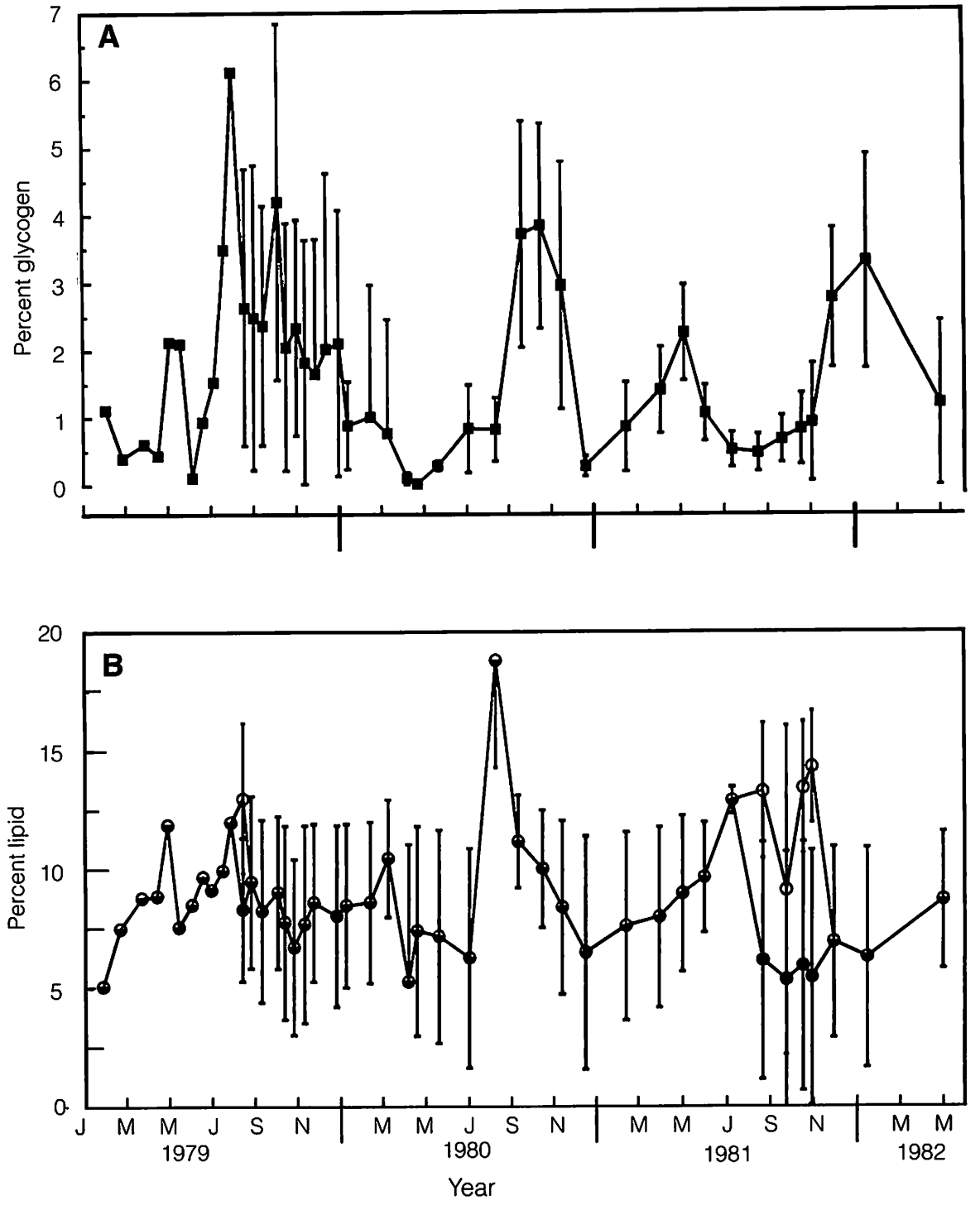

Figure 4. Seasonal variation over a four-year period in the proximate composition of tissue for a cohort (settlement time summer 1978) of *Crassostrea gigas* in the bay of Marennes-Oléron, France. (A) Percent glycogen of male and female oysters combined (■). (B) Percent lipid content of male and female oysters combined (◒) and separate (females ○; males ●). Values are means (± [units of variance estimate not stated]; N = 10 for combined values and N was not stated for the separate sexes). Redrawn from Deslous-Paoli and Héral (1988).

large amounts of the PUFAs 16:1ω7, 20:5ω3, and 22:6ω3 were released with the eggs.

Intraspecific Variation

Several of the studies on energy storage cycles in oysters allude to intraspecific variation, either between populations (Masumoto et al. 1934; Chipman 1948; Walne 1970; Sidwell et al. 1979; Whyte and Englar 1982; Perdue and Erickson 1984) or between years (Chipman 1948; Engle 1951; Deslous-Paoli et al. 1982a; Deslous-Paoli and Héral 1988). The lack of proper replication or variance estimates in many of these papers means that it is difficult to determine whether such intraspecific variation is real. Perdue and Erickson (1984) compared two separate stocks of Pacific oysters and one stock of Suminoe oysters, *Crassostrea ariakensis*[1] grown together in Washington State. Oysters from all three stocks showed large decreases in carbohydrate concentrations during gametogenesis from May to August, but there was no difference between the two stocks of *C. gigas*, in which carbohydrate decreased from 32% dry weight to between 7 and 10% dry weight. Glycogen concentrations in *C. ariakensis*, however, were consistently higher, falling from 40% dry weight to 20% over the same period, although these oysters failed to spawn.

Considerable year-to-year variation in glycogen concentrations was demonstrated by Chipman (1948) for *C. virginica* in Long Island Sound, where peak values of 33%, 45%, and 21% of total dry weight were recorded in three separate years. Interannual variability in lipid and glycogen concentrations can also be seen in the data of Deslous-Paoli and Héral (1988) for *C. gigas*, but there are no consistent trends (Fig. 4). It is often stated, or is implicit in many studies, that intraspecific variation in carbohydrate and lipid is a function of environmental conditions, particularly food availability. This relationship has not been demonstrated unequivocally for oysters, although it has been found in other bivalves (Thompson and MacDonald 1990). Deslous-Paoli et al. (1982a) recorded differences between two years in the production of juveniles by a population of *C. gigas*. They found that lower reproductive success in one year was associated with a reduced carbohydrate and lipid content in the adults after a spring bloom in which lipid, carbohydrate, and protein in the seston were also low compared with a year in which reproduction was more successful. Although Deslous-Paoli et al. (1982a) strongly suggested that a poor spring bloom in one year resulted in a substantial reduction in the glycogen reserves available to support gametogenesis and in the lipid pool required for oogenesis, thus decreasing observed production of juveniles, there were no statistical analyses to validate this hypothesis. It has been shown, however, that carbohydrate concentrations in *C. gigas* and the European flat oyster, *Ostrea edulis*, can be enhanced by providing more food to oysters held at optimum temperatures (Mann 1979).

Given the degree to which glycogen accumulation in oysters appears to depend on ambient food and other environmental conditions, it is not surprising that the glycogen values reported in the literature vary widely, notwithstanding the decreases that occur during gametogenesis. Data from the Pacific oyster illustrate this well. Deslous-Paoli et al. (1982b) recorded glycogen values of 2 to 11% dry weight in *C. gigas* from non-tidal ponds in western France, with glycogen accounting for ca. 95% of the carbohydrate. In *C. gigas* from the nearby Bay of Marennes-Oléron, however, Deslous-Paoli and Héral (1988) reported values of 0 to 7% dry weight for glycogen, depending on the time of the year, with glycogen comprising only half the carbohydrate present. The authors attributed the low glycogen values to high stocking densities in the Bay, which may have resulted in food limitation. In contrast, Whyte and Englar (1982) reported that carbohydrate, of which 79% was glycogen, varied from 25 to 37% dry weight in *C. gigas* grown on- or off-bottom in British Columbia.

According to our calculations from the data of Masumoto et al. (1934), glycogen in *C. gigas* from Kisatsu, Japan, varied from a maximum of 23% dry weight in March and April (Fig. 3A) immediately before spawning, to a minimum of 2% dry weight after spawning was complete in August. A similar trend was observed by Perdue et al. (1981) and Perdue and

[1] Perdue and Erickson (1984) originally identified this species as *C. rivularis*, but the editors have renamed it here after the convention of Carriker and Gaffney, Chapter 1.

Erickson (1984) for *C. gigas* in Washington State, although here the maximum was 30% dry weight and the minimum was 1 to 2% dry weight. In an experimental study (Mann 1979), the glycogen concentrations attained by *C. gigas* were temperature dependent, values as great as 34% dry weight being recorded; a similar trend was observed in *O. edulis*. Carbohydrate concentrations also vary considerably in *C. virginica*, e.g., concentrations range from minimum values as low as 3% dry weight to maximum values as high as 45% (Chipman 1948; Engle 1951) and from 18 to 38% (Fig. 2; Barber et al. 1988b). In a seasonal study of blood metabolites in *C. virginica* (shell height >4 cm) from two populations in the Choptank River, Maryland, Fisher and Newell (1986) found that blood carbohydrate and protein concentrations were higher in one population than the other throughout the year, although the physiological basis for this difference was not ascertained.

The amount of glycogen reserves used to supply energy for reproduction can be estimated in a few studies where data are presented for the total weight of a given biochemical component, rather than for the concentration alone. In other studies, values for total weight can sometimes be calculated from the data given. From the data of Masumoto et al. (1934), we can calculate the net loss of glycogen during gametogenesis and spawning in *C. gigas*, because the reproductive cycle shows clearly defined phases of fattening, gametogenesis, and spawning, which are not evident in many other data sets. The full complement of glycogen appears to be present before gametogenesis begins. Thus, by subtracting the weight of glycogen remaining when spawning begins (June) from the weight present at the beginning of the gametogenic phase (April), we can obtain an approximate estimate of the net loss due to gametogenesis alone, bearing in mind that the processes of glycogen synthesis and utilization are coupled and that glycogen synthesis may continue during the gametogenic phase. In four of the conditions considered in this reciprocal transplant study (Fig. 3, legend), oysters of mean dry weight 1.16 g (range of group means, 1.03 to 1.28 g) lost 0.106 g glycogen (grand mean; range of group means, 0.097 to 0.115 g). During July and August, a further 0.151 g glycogen was lost, most of which was presumably catabolized and the remainder (probably very little) released in the gametes. A similar calculation applied to the data of Allen and Downing (1986) demonstrates that diploid *C. gigas* grown in California showed a net loss of 0.37 g glycogen per month in May and June, during which time the oysters underwent gametogenesis very rapidly (Fig. 5). This is ten times the value of 0.035 g per month obtained from the data of Masumoto et al. (1934), although the oysters from Japan were much smaller (1.16 g dry weight) than the California oysters used by Allen and Downing (1986) (4 g dry weight), and took longer to complete gametogenesis. When compared on the basis of the total consumption of glycogen during the gametogenic period, however, the two groups were similar, because the Pacific oysters in the study of Masumoto et al. (1934) lost 21.5% of their body weight as a result of glycogen depletion, and those used by Allen and Downing (1986) lost 18.5%.

Glycogen Concentrations in Triploid Oysters

Allen and Downing (1986) demonstrated that the carbohydrate concentration in diploid oysters (*C. gigas*) decreased from 26% dry weight at the beginning of gametogenesis to 5% immediately before spawning two months later (Fig. 5). During the same period, carbohydrate concentration in triploids showed an initial increase before decreasing slightly to 26% dry weight when the diploids spawned. The difference must, in part, reflect a much greater metabolic demand by diploid oysters undergoing more intense gametogenic activity than in the reproductively sterile triploids, as well as the synthesis of egg lipids in the diploids. Whereas carbohydrate concentrations in diploid oysters began to recover immediately after spawning, those in triploids continued to decrease for another month after spawning was complete. The authors suggested that this difference may be attributable to the presence of large numbers of undifferentiated cells in the gonads of the triploids, thereby disturbing the endocrine regulation of glycogenesis and glycolysis.

Effects of Environmental Stress Factors

The effects of factors that can be considered as stressors, including fluctuations in environmental conditions, on the synthesis and use of energy reserves has been extensively studied in the blue mussel *M. edulis* (Gabbott 1976, 1983), and to a lesser extent in the sea scallop, *Placopecten magellanicus* (Thompson and MacDonald 1990). In general, carbohydrate reserves are catabolized as an initial response to stress, although protein may be broken down if carbohydrate becomes exhausted and adverse conditions persist. There is little information of this nature for oysters, although we have already noted that glycogen synthesis may be inhibited at low temperature in *C. virginica* (Ruddy et al. 1975), probably because the oyster ceases to feed. Mann (1979) established that temperature plays a role in glycogen storage in *C. gigas* and *O. edulis*, and noted that carbohydrate reserves can act as a buffer against stress.

Riley (1975) demonstrated that starvation of *C. gigas* resulted in a decrease in carbohydrate concentration (expressed as a percentage of dry weight) but no change in lipid concentration. Although more carbohydrate was catabolized, calculations from tissue weight loss showed that lipid yielded more energy, owing to its greater energy content per unit weight. During the initial phases of starvation, the digestive gland and mantle were the primary sources of energy, whereas reserves from the gonad were used extensively in the later stages. This study is one of very few in which weight changes in substrates, rather than concentrations alone, were measured in any species of *Crassostrea* and in which several tissues were analyzed separately.

Other stressors, such as parasitic infection, can also alter the biochemical composition of bivalve tissues through competition for nutrients and by direct disruption of physiological processes, such as feeding activity (Newell and Barber 1988). Parasites such as the trematode *Bucephalus* sp. are known to reduce the concentrations of lipid (Cheng 1965) and carbohy-

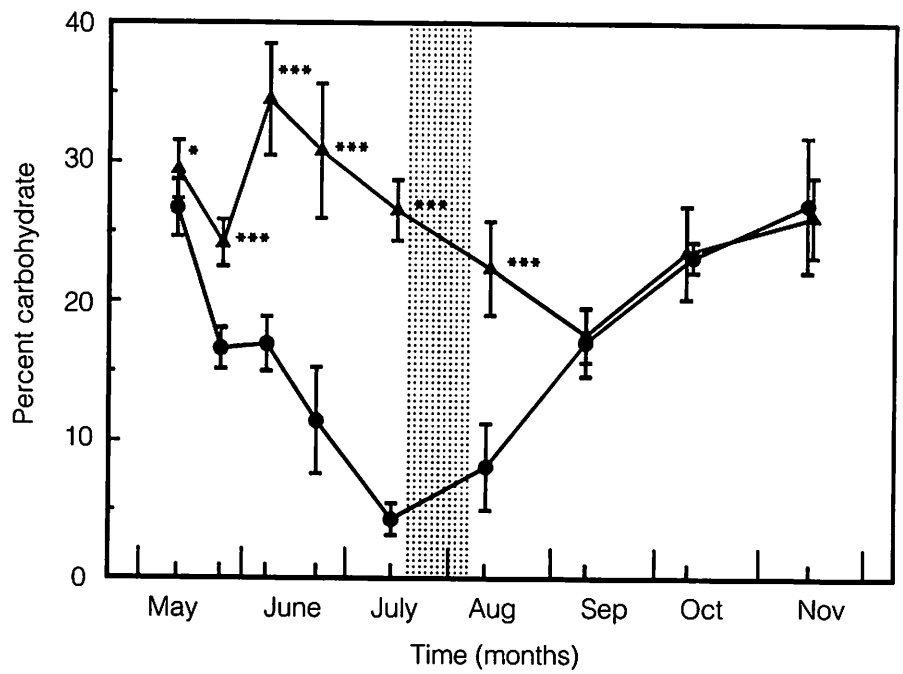

Figure 5. Seasonal changes in carbohydrate content (mean % dry tissue weight [±95% confidence intervals]) in diploid (●) and triploid (▲) *Crassostrea gigas* from Washington State, USA, during the summer. Spawning period for diploid oysters indicated by stippled bar. Means significantly different at * $P < 0.05$ and *** $P < 0.001$. Redrawn from Allen and Downing (1986).

drate (Cheng and Burton 1966) in the eastern oyster. The weights of lipid, protein, and carbohydrate per unit shell size in eastern oysters were shown by Barber et al. (1988b) to be inversely related to the intensity and duration of parasitic infestation by *H. nelsoni* (MSX disease). The decline in carbohydrate content was significant both in the early stages of MSX infestation, where the parasite is confined to the gill epithelium, and also in advanced systemic in festations (Barber et al. 1988b). Protein was only significantly reduced in oysters with systemic MSX infestations. This parasite-induced stress was also shown by Barber et al. (1988a) to have a direct effect on reproductive output. Eastern oysters with epithelial infestations had a condition index (amount of tissue per unit shell volume) and reproductive output 13% and 31% lower than values for uninfected oysters, respectively. In oysters with systemic MSX infestations, the condition index and reproductive output were 31% and 81% lower than in uninfected oysters, respectively. The decrease in fecundity was manifest primarily as a reduction in the number of eggs produced and not as a decrease in egg size. This suggests that because the parasitic stress was imposed before the initiation of gametogenesis, oysters could compensate by producing fewer eggs, but those eggs still would produce viable larvae.

Ford and Figueras (1988) and Ford et al. (1990) determined that gametogenesis was inhibited during the spring in direct proportion to MSX infestation intensity. However, during the summer, when high water temperatures reduced the intensity of the parasite, many MSX-infested oysters subsequently developed at least some mature gametes that were spawned. Kennedy et al. (1995) reported that the size of the gonad in oysters from Chesapeake Bay was reduced in direct proportion to the degree of infection by the protozoan *Perkinsus marinus* during the period of peak reproductive condition just before spawning. The eggs spawned by infected oysters, however, were of the same size and contained the same amounts of lipid reserves as those spawned from uninfected oysters. Choi et al. (1993) did not find a similar inhibition of reproductive condition in oysters from the Gulf coast infected by *P. marinus*. Choi et al. (1994) did report, however, that heavy *P. marinus* infections retard the rate of gamete development during the fall, but do not influence the spring spawning period in eastern oysters from the Gulf coast.

The capacity of parasitized eastern oysters to reduce the numbers of eggs produced but maintain the normal egg size and composition is similar to the reproductive compensations exhibited by other species of bivalves when nutrient reserves are reduced by environmental factors, such as increases in temperature or reductions in food availability. For example, Helm et al. (1973) demonstrated for *O. edulis*, and Bayne et al. (1978) for *M. edulis*, that if the stress occurs early in the reproductive cycle there is a reduction in numbers of eggs produced, although the eggs are normal in size and lipid composition and hatch to produce viable larvae. In contrast, when the females are stressed after the onset of gametogenesis and the initial stages of egg production, vitellogenesis is inhibited and the eggs have smaller yolk reserves and produce larvae with reduced viability. The similarity in response between different species of bivalve exposed to various stress factors suggests that this is a general adaptive mechanism whereby bivalves compensate for reductions in nutrients available for gametogenesis.

FECUNDITY AND REPRODUCTIVE EFFORT

Reproductive output and reproductive effort of bivalves are important quantities in that, like growth, they are likely to respond to environmental conditions and to represent important fitness correlates. Many of the published estimates of fecundity in *C. virginica* are not related to the tissue weight of the animal, so that meaningful comparisons among studies are not possible. The determination of fecundity in non-brooding oysters presents some difficulties because the gonad is diffuse and invades other tissues, precluding most procedures that are based on weighing the gonad. A further difficulty arises in that many oysters, including *C. virginica*, may spawn repeatedly throughout a single season or year, so that an estimate of the gametes present in the gonad, or released at any given time, does not necessarily represent the entire annual reproductive output.

Histological techniques have been developed that allow both seasonal changes in reproductive condition and relative estimates of reproductive output in oysters to be made (Mori 1979; Perdue et al. 1981; Barber et al. 1988a, 1991; Kennedy et al. 1995). The optimum position for sectioning the visceral mass of eastern oysters to assess their reproductive condition is at the junction of the gills and labial palps (Morales-Alamo and Mann 1989), because the gonad does not form a complete circle around the digestive gland at the extreme dorsal and ventral positions of the visceral mass. The basis of this method is that the area or width of somatic tissue is conservative, but that as the oysters become more gravid and then spawn, the width of the germinal tissue surrounding the somatic tissue will first expand, then contract. A reproductive index for both male and female oysters is then calculated by expressing the area of germinal tissue as a percentage of the area of the entire visceral mass (e.g., Fig. 2).

Literature on absolute fecundity and reproductive energetics of oysters is not extensive. Although there are some estimates of fecundity, there have been very few attempts to integrate them with growth and physiological studies to determine indices such as reproductive effort, which provides a measure of the relative amounts of energy allocated to growth and reproduction. In contrast, the fecundity or reproductive output data for blue mussels (reviewed by Seed and Suchanek 1992) and for various species of scallops (reviewed by Thompson and MacDonald 1991) are often more focused and easier to interpret.

Galtsoff (1930) counted the eggs released by individual eastern oysters and found that a single female could produce from 15 to 115 million eggs at one spawning. He estimated that as many as 500 million eggs may be spawned by a female during the season, but given the uncertainties associated with such estimates and the lack of detail provided, this figure has been questioned by subsequent authors. Later, Galtsoff (1964) reported values of 10 to 20 million eggs as typical for a single spawn, with occasional spawnings of as many as 100 million. Direct counts were also made by Davis and Chanley (1956), who obtained fecundity values for oysters from Connecticut of 10,000 eggs to 66 million eggs per spawning, depending on body size, which they recorded in terms of shell-cavity volume.

Cox and Mann (1992) used a different procedure to estimate fecundity in *C. virginica* from four oyster beds in the James River, Virginia. They homogenized the soft tissues of individual oysters collected at intervals throughout the reproductive season, screened out the debris, and counted the eggs and oocytes that remained. Values obtained by this method may be regarded as estimates of potential fecundity. The mean fecundity was 4 to 9 million eggs per female, depending on body size and the sampling site. These values are lower than those obtained by other authors who induced oysters to spawn, but this is attributable to both mechanical destruction of eggs and the inclusion in the samples of oysters that were at various stages of development, including some which may have already spawned or spawned only partially. In order to overcome the inclusion of partially spawned and non-gravid oysters, we reanalyzed the data of Cox (1988) using only the highest fecundity estimate for females from each weight class. A power curve was then fitted to relate the number of eggs to dry tissue weight (Fig. 6). This analysis yields fecundity values varying from 2 million eggs for an oyster of 0.3 g dry weight (about 4 cm long) to 45 million for one of 1 g dry weight (about 7 cm long). The exponent of 2.36 for the power function (Fig. 6) emphasizes that as eastern oysters grow larger, they divert a greater proportion of their assimilated food to egg production. Similarly, Dame (1976) reported that germinal production, measured as the weight of excised gonadal material, is related to shell size of *C. virginica* from South Carolina by a power function with an exponent of 2.89 and 2.60 for females and males, respectively. Rodhouse (1978) also demonstrated that in the European flat oyster, energy is initially allocated to somatic growth, but as the oysters grow older there is an exponential increase in the amount of energy allocated annually to germinal production.

The literature on size-related energy partitioning in bivalve molluscs has been reviewed by Thompson and MacDonald (1991). Petersen (1983) reported

that in the hard clam *Mercenaria mercenaria* the exponent of the power curve relating gonadal mass to shell length was significantly greater than 3. This indicates that gonadal mass increased at a faster rate than length cubed and means that larger clams devote an increasingly larger proportion of their body tissue to germinal production. Future studies, in which such measurements of individual fecundity are integrated with field data on population size structure, are required to estimate the relative importance of large and small oysters to the total fecundity of natural eastern oyster populations.

Mann et al. (1994) extended their observations on oysters in the James River, Virginia, and compared values between populations and between years. For two sites (Horsehead Reef and Wreck Shoal), fecundity was significantly different over three years, with annual mean values per female of 5.6 million, 0.94 million, and 0.12 million eggs, respectively. This gradual reduction in fecundity was correlated with declining average salinities. The stress of low salinity may have reduced the energy available to these oysters to allocate to reproduction (Butler 1949) but the environmental factors underlying the variation between populations and years are generally not well understood. It is apparent, however, that *C. virginica* exhibits intraspecific variation in fecundity between "populations" on a small spatial scale, and that fecundity may vary from year to year in a given population, as has been shown for the sea scallop,

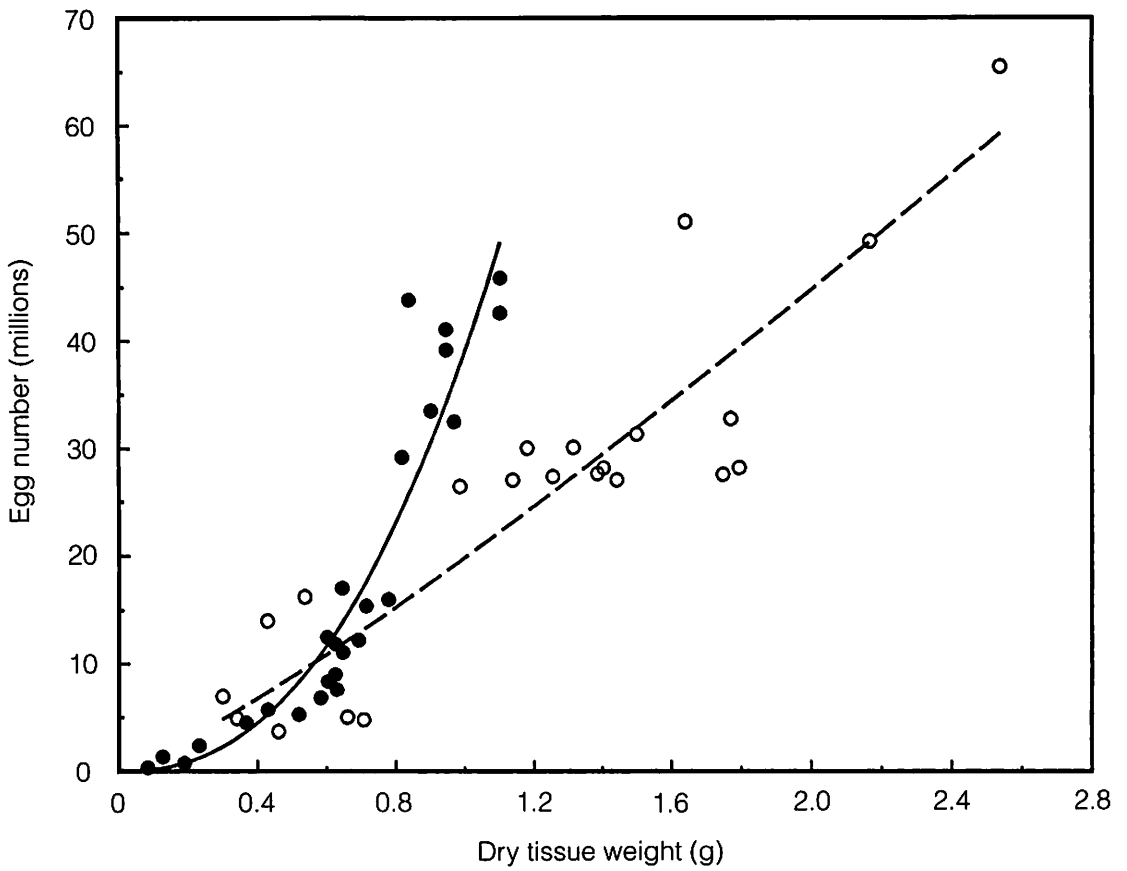

Figure 6. Number of eggs produced by eastern oysters from the James River, Virginia (●), and West Bay, Texas (○), as a function of dry tissue weight (g). The variables (± SE) for the fitted power curve for the data from the James River population (Cox 1988) are $Y = 39.07\ (\pm\ 1.94) \times X^{2.36\ (\pm\ 0.24)}$; [N = 26; R^2 = 0. 89]. The variables (± SE) for the fitted power curve for the data from the West Bay population (Choi et al. 1993) are $Y = 19.86\ (\pm\ 1.86) \times X^{1.17\ (\pm\ 0.15)}$; [N = 22; R^2 = 0. 83].

Placopecten magellanicus (Thompson and MacDonald 1991).

In order to overcome the technical problems described above for estimating fecundity in eastern oysters, Choi et al. (1993) developed an enzyme-linked immunosorbent assay that allows both egg and sperm production to be quantified more accurately. This method, although technically more complex than older techniques, allows accurate comparisons of fecundity among oyster size-classes and oyster populations in different habitats. Choi et al. (1993) used the technique to estimate the fecundity of 43 female oysters from Texas ranging in tissue dry weight from 0.3 to about 2.8 g, including some that had partially spawned. As discussed above for the study of Cox (1988), the inclusion of such data from partially spawned oysters will cause total fecundity to be underestimated. To overcome this problem we have replotted in Figure 6 the data from Choi et al. (1993) for the oysters that had fecundity values greater than the mean for that weight class. This analysis yields fecundity values varying from 5 million eggs for an oyster of 0.3 g dry weight to 20 million for one of 1 g dry weight. The fecundity of eastern oysters from the Gulf coast does not increase as much with size as in oysters from Chesapeake Bay (Fig. 6). Indeed, egg production increases almost linearly with body weight (exponent of the power curve in Fig. 6 is only 1.17) in Gulf coast oysters. This relationship is more similar to data from species of brooding oysters in which reproduction is limited by the volume of the mantle cavity, where the lavae are brooded (Fig. 7).

Choi et al. (1993) postulated that the relatively low reproductive output of the larger oysters from the Gulf coast may be indicative of "reproductive senility" (*sensu* Petersen 1983). They also suggested that larger oysters may have a reduced rate of net energy assimilation compared to smaller ones. This reduction would cause larger oysters to attain peak spawning condition later in the spring and increase the time required to develop new gametes after initial spawning. Choi et al. (1993) based both of their explanations on predictions from a physiological model developed by Powell et al. (1992) and Hoffman et al. (1992). This model is predicated on the assumption that larger oysters should have less energy to devote to reproduction because they have reduced feeding rates and higher metabolic rates compared to smaller oysters. This balance between food intake and metabolic rate changes with body weight because in most bivalves that have been studied the slope of the allometric relationship relating feeding rate to body weight is lower than that relating metabolic rate to body weight (Bayne and Newell 1983; Thompson and MacDonald 1991). The physiological model developed by Powell and coworkers may be biased, however, because it simulates very rapid early growth of oysters, so that most oysters enter the heaviest size classes (> 2 g ash free dry tissue weight) by the time they attain one year of age. It is only in these heaviest oysters that metabolic energy demands start to become a disproportionately large component of consumed energy. In fact, only 10% of the oysters collected by Choi et al. (1993) exceeded 2 g dry tissue weight, making implausible their suggestion of energy limitation of germinal production as a general explanation of the linear relationship between reproductive output and body weight. Indeed, the two proposals of Choi et al. (1993) are inconsistent with studies on other oyster populations (discussed above). Furthermore, in the literature reviewed by Petersen (1983), there was no evidence that any species of bivalve mollusc exhibits reproductive senility. Since then there has been evidence of declining reproductive production in larger individuals only in two species of scallop, *Chlamys islandica* (Vahl 1985) and *Argopecten irradians irradians* (Bricelj and Shumway 1991).

A more plausible explanation for the relatively low fecundity of larger oysters from the Gulf coast may be that all the oysters used by Choi et al. (1993) were infected with *P. marinus*. This parasite is known to reduce both the rate of gametogenesis (Choi et al. 1994) and total fecundity (Kennedy et al. 1995) of eastern oysters. However, Cox and Mann (1992) reported that the populations from which oysters were collected by Cox (1988) to estimate the fecundity of Chesapeake Bay oysters were also infected by both *P. marinus* and *H. nelsoni*. Nevertheless, oysters from this collection (Fig. 6) did not exhibit any apparent decline in reproductive output with size. Further studies are required to determine if parasitism or some other factor was contributing to the compara-

tively low fecundity of larger oysters in the Gulf coast.

In some species of ostreids, eggs are not released into the water column but are fertilized within the gills by sperm brought in with the mother's feeding currents. The embryos and larvae are then brooded for a portion of the development period, thereby increasing larval survival. These higher survival rates mean that female oysters are able to allocate less energy to egg production. In these oyster species the measurement of fecundity is more straightforward because accurate direct counts of numbers of larvae can be made. This is reflected in the literature, where the variance is typically greater for fecundity estimates in *Crassostrea* spp. than in *Ostrea* spp. In brooding oysters, however, the measurement of annual reproductive output at the level of both the individual and the population is complicated by observations that an individual female may produce more than one brood per year and that not all females in a population necessarily brood in any given year (Cranfield and Allen 1977; O'Sullivan 1980). Normally one assumes that the number of larvae incubated is equivalent to the number of eggs released, although this may lead to an underestimate of fecundity if the fertilization rate is less than 100%.

Walne (1964) related numbers of larvae incubated by individual *O. edulis* from two populations to various correlates of body size, including shell length and tissue dry weight. There was very little difference between populations, but a strong relationship with

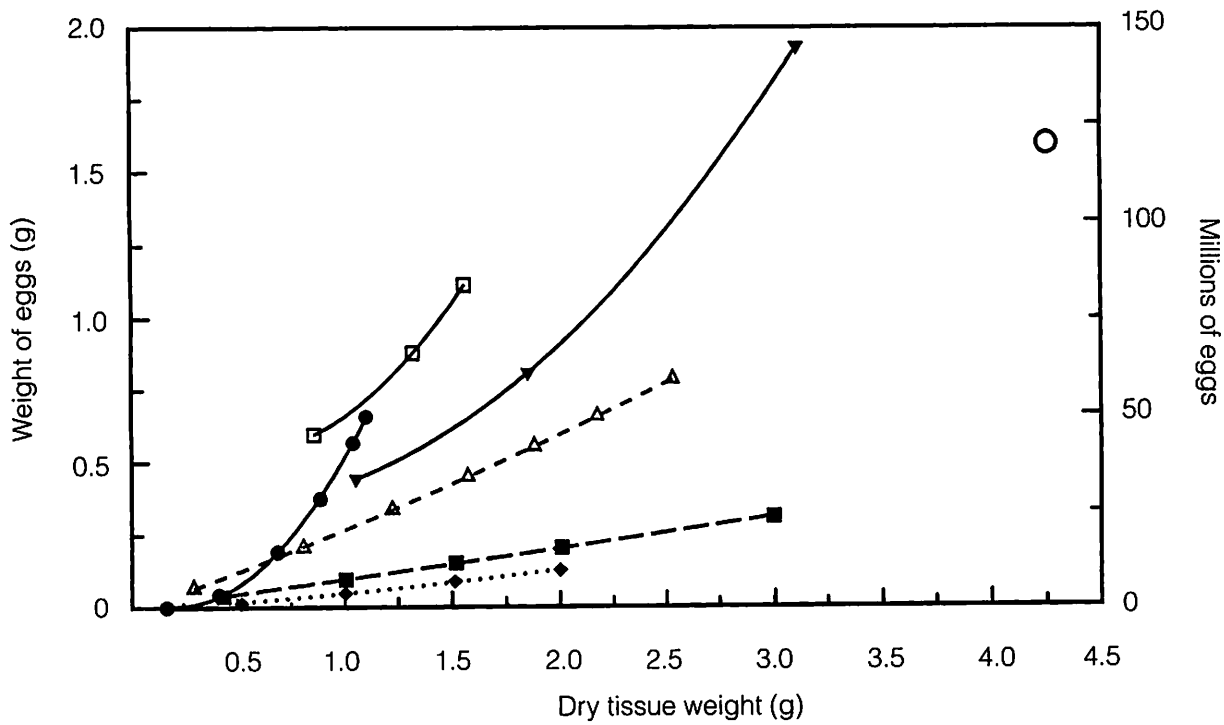

Figure 7. Weight (g) of eggs spawned (left axis) and millions of eggs spawned (right axis [only for *Crassostrea* species]) as a function of prespawning soft-tissue dry weight for a variety of ostreid species. Data are presented for the actual ranges of body weight present in the source data sets. For the following three data sets for *Crassostrea gigas*, we calculated group means at the specified body weight. □ (Kusatsu oysters held at Kusatsu; Masumoto et al. 1934); ▼(Deslous-Paoli and Héral 1988); O (diploid Pacific oysters; Allen and Downing 1986). For the remaining data sets we fitted lines to discrete data points. ● (*Crassostrea virginica* from the James River, Virginia; Cox and Mann 1992 and Mann et al. 1994); △ (*Crassostrea virginica* from West Bay Texas; Choi et al. 1993); ■ (*Ostrea edulis*; Walne 1964); and ◆(*Ostrea puelchana*; Cranfield and Allen 1977).

size. Females of 1 g dry weight incubated about 0.7 million larvae, and those of 2 g dry weight about 1.3 million. Variation in fecundity between individual oysters was associated with condition, those with a higher condition index incubating more larvae. Rodhouse (1978) measured reproductive output in *O. edulis* from a single population by two methods: one indirect, in which the weight loss on spawning was recorded, and the other direct, in which the freshly released eggs were collected from the mantle cavity and the energy content determined by bomb calorimetry. Assuming that each egg is 120 μm diameter and has an energy content of 41.5×10^{-4} J (calculated from a value of 23 kJ g^{-1} dry weight obtained by Bayne et al. [1975] for eggs of *M. edulis*), we can derive fecundity values ranging from 0.4 to 1.4 million eggs for *O. edulis* of 50 mm shell height, to 2.6 to 3.2 million at a shell height of 80 mm. These estimates are two or three times greater than those of Cole (1941) and Walne (1964) for *O. edulis* of comparable shell size.

Cranfield and Allen (1977) found that in New Zealand the flat oyster, *Ostrea puelchana*,[2] also showed a strong correlation between fecundity and body size. In this species, an individual weighing 1 g dry weight brooded 50,000 larvae, which is equivalent to 635,000 larvae of *O. edulis* (the egg of *O. puelchana* is 12.7 times the volume of an egg of *O. edulis*; 280 μm diameter compared with 120 μm). Thus, the two species have the same reproductive output in terms of the weight of eggs released (Fig. 7), if we use data on *O. edulis* from Cole (1941) or Walne (1964) for comparison. The values for *O. puelchana* from New Zealand are very similar to those quoted by Walne (1963) for the (putative) same species in Chile.

The gametogenic cycles of *C. gigas* at the sites used by Masumoto et al. (1934) and Deslous-Paoli and Héral (1988) are discrete, and the published data allow us to calculate growth and reproductive output, and hence reproductive effort (Table 3). The weight loss on spawning is a measure of reproductive output, and the net change in tissue weight over a twelve month period is an estimate of somatic production. Both quantities may be expressed in energy terms, for which we have assumed a value of 23 kJ g^{-1} dry weight for eggs (Bayne et al. 1975) and 21.8 kJ g^{-1} dry weight for somatic tissue (Bayne et al. 1975). We calculated the number of eggs released (fecundity) by assuming an egg diameter of 50 μm and a density of unity. The dry weight of the oyster immediately before spawning was used as a measure of size. The Pacific oyster devotes a large part of its available energy to reproduction, and in larger and older individuals this reproductive effort exceeds 80% (Table 3). The transition from growth to reproduction as the individual ages is evident in the data of Deslous-Paoli and Héral (1988). Similar trends have been observed in other oviparous bivalves (Thompson and MacDonald 1991).

Comparing Walne's (1964) values for the fecundity of *O. edulis* (Fig. 7) with the above data for *C. gigas*, we find that a Pacific oyster of 1 g dry weight loses about 15 kJ during the spawning period (although not necessarily in a single spawning), and one of 1.5 g dry weight loses about 25 kJ, whereas European flat oysters of the same weight lose 2.32 kJ and 3.45 kJ, respectively. There is a difficulty in comparing the two species, because *O. edulis* may spawn two or three times in the season depending on conditions (Cranfield and Allen 1977; O'Sullivan 1980), or may not spawn at all. Furthermore, there is a possibility of underestimating the fecundity of *O. edulis* because some of the eggs may be lost from the mantle cavity before brooding begins and before the larvae are counted. Notwithstanding these complications, the available information suggests that the reproductive output of *O. edulis* is lower than that of *C. gigas* and of *C. virginica*, and gamete production in *O. puelchana* is lower than in any ostreid for which there are data (Fig. 7). This may simply be because the total number of larvae brooded is limited by space within the mantle cavity. On the other hand, brooding behavior may impose reproductive costs on the parent, such as a higher metabolic rate and possible interference with the feeding process, costs that are not incurred by broadcast spawners such as *Crass-*

[2] Note that the taxonomic status of austral ostreid species is controversial. The authors originally identified this species as *O. lutaria*, but the editors have renamed it here after the convention of Carriker and Gaffney, Chapter 1.

ostrea spp. At present we know of no data from which the process of reproduction in *C. virginica* can be analyzed in a similar fashion.

FACTORS INFLUENCING SPAWNING

Manipulation of water temperature has long been recognized as a means of stimulating ripe eastern oysters to spawn under hatchery conditions (Loosanoff and Davis 1963). Data pointing to temperature as an environmental stimulus inducing mass spawning in natural oyster populations, however, are equivocal. Do oysters spawn as a result of a slow, steady temperature increase to a critical threshold, or as a result of a rapid change after a certain gametogenic condition is attained? Medcof (1939) considered that spawning is preceded by sudden rises in water temperature, and Butler (1956) postulated that the rate of temperature change is more important than some "critical" level being attained. Similarly, Hayes and Menzel (1981) proposed that although Gulf Coast oysters only spawn in the spring after water temperatures attain 25°C, the stimulus for spawning in the fall is a sharp decline in water temperature that may occur as early as late July to early August. Unfortunately, because of the uncontrolled nature of these field observations it is difficult to ascertain the cause-and-effect relationship between changes in water temperature and the general biological response of spawning.

A number of molluscs and other invertebrates spawn in response to various natural substances present in seawater, with ectocrines derived from algae apparently a particularly potent spawning stimulus (for reviews see Himmelman 1981; Giese and Kanatani 1987). Galtsoff (1938) found that such chemical stimuli were even more effective than temperature change in triggering spawning of eastern oysters in the laboratory. Miyazaki (1938) reported that male Pacific oysters spawned when exposed to an unknown substance derived from the macroalga *Ulva* sp. Breese and Robinson (1981) stimulated a number of bivalve species, including *C. gigas* and *C. ariakensis*, to spawn by exposing them to several phytoplankton species, although other methods of eliciting spawning (temperature change, chemical stimulation) were unsuccessful. Similarly, in *C. virginica* (Galtsoff 1964) and *M. edulis* (Starr et al. 1990), males can be triggered to spawn by a chemical agent released by phytoplankton. Because male blue mussels often spawn earlier than females, Starr et al. (1990) postulated that phytoplankton stimulate the most responsive males to spawn first, their sperm and the available

Table 3. Reproductive effort for Pacific oysters of different weights calculated from literature data on annual cycles in tissue weight. For the data from Deslous-Paoli and Héral (1988), the three weight groups are equivalent to 1-, 2-, and 3-year old oysters, respectively.

Mean dry tissue wt (g)	No. eggs (millions)	Energy content of gametes (kJ)	Somatic energy increment (kJ)	Total energy production (kJ)	Reproductive effort
		Masumoto et al. (1934)			
0.85	45	13.6	2.4	16.0	0.85
1.30	67	20.0	5.0	25.0	0.80
1.54	85	25.5	4.4	29.9	0.85
		Deslous-Paoli and Héral (1988)			
1.05	34	10.3	12.6	22.9	0.45
1.85	62	18.6	2.6	21.2	0.88
3.10	148	44.4	4.4	48.7	0.91

phytoplankton then inducing mass spawning by the rest of the population. Thus, spawned eggs are released into water containing active sperm, with fertilization reasonably assured. The chemical nature of the ectocrines responsible for these responses in bivalve molluscs and their presence and activity in nature remain to be fully elucidated. Starr et al. (1992) partially purified from the unicellular algae, *Phaeodactylum tricornutum,* a phenolic compound that stimulates spawning in the green sea urchin, *Strongylocentrotus droebachiensis.* It is likely that this same compound is also effective in triggering spawning of bivalve molluscs (Starr et al. 1992).

Once spawning has been initiated, perhaps by the presence of algal ectocrines, the stimulatory role of gametes on spawning in both sexes ensures a synchronized mass spawning. Galtsoff (1938) determined that in eastern oyster sperm the active component responsible for stimulating female oysters to spawn is species specific, whereas in the eggs the active compound that stimulates male oysters to spawn is common to eggs of other species of molluscs (Galtsoff 1940). Galtsoff (1938) also showed that a similar response could be induced by a number of chemical compounds. Further research has shown that serotonin (5-hydroxytryptamine) is an important neurotransmitter involved in the spawning process of male (and, to a lesser degree, female) bivalve molluscs (Matsutani and Nomura 1982), including eastern oysters (Gibbons and Castagna 1984). In addition to the important role that this compound plays in natural spawning, its stimulatory effect has been exploited to induce spawning of male eastern oysters in commercial aquaculture (see Castagna et al., Chapter 19).

Variations in salinity do not appear to play a role in stimulating spawning in the eastern oyster, although salinities below 5 or 6 ppt can inhibit gametogenesis (Butler 1949; Loosanoff 1953). However, spawning in response to a salinity decrease has been reported in some oyster species that live in monsoon climates (Durve 1965; Joseph and Madhyastha 1984). Salinity requirements in these instances probably reflect the sensitivity of the larvae rather than that of the generally more resistant adult (Wilson 1969).

In general, the literature shows that temperature (Nelson 1928a, b), or a food-related stimulus (Nelson 1955, 1957), or both, may be responsible for initiating spawning.Starr et al. (1990) suggested that because water temperature can vary unpredictably, a phytoplankton bloom is a more reliable spawning cue and is indicative of the most favorable conditions for planktotrophic larvae in the water column. Whether or not temperature or food stimulate spawning, a critical density of oyster broodstock is clearly necessary to ensure both a coordinated spawning response to stimulation and an optimal concentration of sperm for successful fertilization (Hancock 1973; also see below). It should also be emphasized that we do not know how changes in external or internal factors initiate or control the processes of spawning (Fig. 1). Although serotonergic innervation appears important, the receptors or effectors involved in spawning have not been identified. The exogenous and endogenous control of spawning require further research before we can fully understand the reproductive adaptations of the eastern oyster.

SUCCESS OF REPRODUCTION

Fertilization Success

The production of large numbers of gametes by broadcast spawners, such as the eastern oyster, is generally thought to be an adaptation to compensate for losses associated with a planktonic existence. In such species, fertilization success is influenced by numerous factors, including the viability of the gametes. Nelson (1891) reported that sperm collected from freshly opened oysters could remain viable for as long as 5 h, but that eggs lost their ability to be fertilized about 1 h after their separation from ovarian tissue. Galtsoff (1964) found that sperm in a dilute suspension at room temperature could retain their fertilizing capability for 4 to 5 h, but that eggs remained viable for longer periods than was suggested by Nelson (1891).

These data on fertilization success in eastern oysters can be compared with those determined for oth-

er oyster species. Santos and Nascimento (1985) observed that in the mangrove oyster *C. rhizophorae*, 50% of the eggs that were fertilized within 45 min of sperm collection produced normal D-hinge larvae after 24 h, compared with 35%, 25%, and 16% when fertilization occurred 60, 90, and 120 min respectively after sperm collection. Helm and Millican (1977) found that if fertilization of Pacific oyster eggs was delayed for more than 90 min, larval development was greatly retarded.

For hatchery production of Pacific oyster larvae, Stephano and Gould (1988) recommended that eggs, whether spawned naturally or surgically removed, be allowed to stand for 1 h before fertilization. They found that such treatment reduced the incidence of polyspermy to < 7%, even at sperm-to-egg ratios up to 1,000. Eggs standing for as long as 105 min did not deteriorate in that they still produced a large proportion (86%) of normal larvae.

These findings that gametes from various species of oysters deteriorate after a few hours underlines the importance of synchronized spawning in nature to maximize the probability of fertilization. As discussed in the section above on "Factors Influencing Spawning," male oysters generally are more responsive than females to spawning stimuli, and it is the presence of sperm that actually stimulates the female to spawn. Stephano and Gould (1988) suggested that in natural populations of oysters the interval between spawning and fertilization is brief. This is because fertilization, even in the non-brooding genus *Crassostrea*, probably occurs within the female's mantle cavity, resulting from sperm brought in by the inhalant water current encountering eggs being discharged through the gills.

Gamete Concentrations

The concentration of gametes in the water also affects fertilization success. Loosanoff and Davis (1963) established that the optimum concentration of suspensions of *C. virginica* eggs for fertilization in the hatchery was 30,000 L^{-1}, but Helm and Millican (1977) reported for *C. gigas* that higher concentrations (up to 100,000 eggs L^{-1}) allowed 80% or more larvae to develop normally to the D-hinge stage. Santos and Nascimento (1985) determined that for hatchery production of normal D-hinge larvae of mangrove oysters, the optimal concentration of eggs was 10,000 to 40,000 L^{-1}, and deleterious polyspermy could be avoided by maintaining sperm concentrations of 500 to 5,000 per egg. Stephano and Gould (1988) used sperm-to-egg ratios up to 1,000 for *C. gigas* and found that more than 85% of the larvae developed normally.

The mechanisms by which an increase in the concentration of sperm per egg produces abnormalities or decreased zygote viability in bivalves are largely unknown. Stiles and Longwell (1973) reported no correlation in eastern oysters between polyspermy (which ranged from 4 to 84% of fertilized eggs in their study) and unsuccessful development, the proportion of abnormal larvae, or settlement success. However, these authors did find that concentrations greater than approximately 20 sperm per egg resulted in significant numbers of chromosome and cell division abnormalities. Stephano and Gould (1988) reviewed this study and suggested that Stiles and Longwell (1973) severely overestimated polyspermy due to methodological problems, including the failure to differentiate between externally attached sperm and those that had penetrated the egg. In their own research with *C. gigas*, Stephano and Gould (1988) reported that eggs penetrated by more than one sperm failed to undergo normal cleavage and that the degree to which larvae developed normally was highly correlated with monospermy.

Whether these hatchery data on sperm and egg concentrations in relation to fertilization have any relevance to field conditions has not been determined. The possible deleterious effects of gamete dilution was shown by Pennington (1985), who found that in the green sea urchin, *Strongylocentrotus droebachiensis*, reproducing under natural conditions, dilution of sperm by fast current flow or by distance from donor males can reduce fertilization success. Similar results were obtained by Levitan et al. (1991), who reported that fertilization success in another sea urchin, *S. franciscanus*, is strongly influenced by the concentration and age of the sperm, and by the duration of contact between the gametes.

Data similar to those of Pennington (1985) or Levitan et al. (1991) are unavailable for the eastern oyster. At present, overfishing and disease in areas such as Chesapeake Bay are causing large reductions in oyster numbers. Unfortunately, there is no information on the numbers or densities of broodstock required to ensure that spawning and fertilization proceed satisfactorily, although Galtsoff et al. (1930) estimated that a successful spawning bed requires more than about 6×10^4 oysters [>8-cm shell height] per hectare.

Ingestion of gametes by microheterotrophs and metazoan suspension feeders is an additional factor that depletes gamete concentrations in the natural environment. Galvao et al. (1989) found that oyster sperm were rapidly ingested by microprotozoans such as heterotrophic nanoflagellates. Sperm supported protist growth, and there was some evidence that microprotozoans preferred sperm to the less nutrient-rich marsh bacteria. Galvao et al. (1989) estimated that over half the oyster sperm released in a salt marsh could be removed by the resident microbial grazer population in 24 h. Such rapid ingestion would significantly reduce the numbers of oyster sperm, possibly decreasing larval production.

Egg Lipids and Developmental Success

Bivalve eggs contain lipid and protein reserves that are essential for larval development (Bayne 1972; Helm et al. 1973; Bayne et al. 1975). Newly-spawned *C. virginica* eggs are about 50 µm in diameter (Stafford 1913; Andrews 1979; Kennedy et al. 1995). Each egg weighs 12 ng, of which 21% is lipid, consisting of 76% triglycerides and 16% phospholipids, with the balance being sterols (Lee and Heffernan 1991). The highest egg lipid content recorded by Kennedy et al. (1995) for *C. virginica* from Chesapeake Bay (4.6 ng egg^{-1}) is almost double the value of 2.5 ng egg^{-1} measured by Lee and Heffernan (1991) for oysters from Georgia. Notwithstanding possible differences in technique between studies, this suggests that environmental factors are important determinants of egg composition in oysters. Eggs also contain 50% protein by dry weight, the balance being ash (Lee and Heffernan 1991), although Choi et al. (1993) reported that eggs of eastern oysters from Texas contain 40% protein by dry weight.

The number of eggs spawned and the quantity of nutrients sequestered in the egg yolk is dependent on the nutritional status of the female (Sastry 1979; Gabbott 1983). As discussed previously in the section on "Effects of Environmental Stress Factors," the effects of sublethal environmental factors that reduce the quantity of food available to support gametogenesis are dependent on when that stress occurs. If it is before the initiation of gametogenesis, female blue mussels produce fewer eggs (although these are of normal size and biochemical composition) whereas if the stress occurs after the initiation of gametogenesis, the eggs spawned may be smaller than normal because of reduced yolk content (Bayne 1975; Bayne et al. 1978). Such eggs have a lower rate of fertilization and embryogenesis than those spawned by unstressed females, and develop into larvae that exhibit a reduced growth rate (Bayne et al. 1978). Helm et al. (1973) have also shown that nutritionally compromised or stressed *O. edulis* produce larvae with reduced survival and vigor. Such important factors affecting ecological fitness require further study under field conditions to determine how much of the annual variability in larval settlement and recruitment is due to adult and larval nutrition. The development of lipid staining techniques (Gallager and Mann 1986; Castell and Mann 1994) suggest the quantification of lipid reserves in field-collected larvae to be an attainable goal.

Lannan (1980) reported that the variation in survival of *C. gigas* larvae is related in part to the state of gonadal development in the parents at spawning. In turn, this variability in gametogenesis is influenced by genetic and environmental factors. Expanding upon this work, Lannan et al. (1980) discovered that spawning and fertilization that appears to be normal in *C. gigas* does not always result in satisfactory larval survival and settlement in the hatchery. Rather, eggs that provide the greatest settlement success are produced in an "optimal conditioning interval" during gametogenesis. That is, the greatest proportion of eggs that subsequently produce spat are those spawned when the female is ripest. Eggs spawned before or af-

ter this period are less likely to produce larvae that survive, develop, settle, and metamorphose.

Muranaka and Lannan (1984) reported that supplemental feeding of Pacific oyster broodstock with algae increased fecundity (measured as the ratio of the area of a gonad section that contained eggs to the total area of the section), but did not influence gamete viability. More detailed nutritional studies by Robinson (1991) on *C. gigas* have confirmed that broodstock nutrition can influence larval viability, because feeding *C. gigas* var. *kumamoto* on either algae or lipid microcapsules increased both the number of eggs released and the proportion of embryos that successfully developed into straight-hinged larvae. This improvement in reproductive output was only apparent when oysters were brought into the hatchery in spring, before the initiation of vitellogenesis. Food supplements supplied to broodstock after the initiation of vitellogenesis did not increase reproductive output.

Gallager and Mann (1986) found survival and growth of larval *C. virginica* to be influenced by season and the length of the period during which adults were fed an algal diet. Broodstock collected during the winter, before the initiation of gametogenesis, had to be fed for a longer period to obtain maximal larval survival than broodstock obtained after the initiation of gametogenesis. The lipid content of the egg was correlated positively with egg diameter. Larval survival was correlated with the initial amount of total lipid transferred to the eggs during vitellogenesis, but subsequent larval growth was not so correlated. These findings imply that a minimum quantity of lipid (related to egg size) is essential for survival during non-feeding larval stages (usually over the first 24 h of larval life; see Lucas and Rangel 1983), with other non-genetic and genetic factors becoming important subsequently.

Changes in the biochemical composition of *O. edulis* larvae and early spat during development have been described by Millar and Scott (1967), Holland and Spencer (1973), Gabbott and Holland (1973), and Holland and Hannant (1973). When starved for short periods, larvae use lipid (especially neutral lipid) and protein, but not carbohydrate, as respiratory substrates. Most of the neutral lipid reserves are catabolized at metamorphosis. Holland and Spencer (1973) also recorded a considerable loss of phospholipid during starvation of newly-released *O. edulis* larvae, suggesting that phospholipid is used as a metabolic substrate. According to Holland (1978), there was good agreement between determinations of oxygen consumption and metabolic rates calculated from observed losses of biochemical reserves in the starvation experiments.

EGG AND LARVAL DEVELOPMENT

The development of the zygote of the eastern oyster has been described by various investigators (Brooks 1879, 1880; Stafford 1905, 1909, 1910). Details of cell division and cleavage are provided by Galtsoff (1964) and Sastry (1979). Elston (1980) undertook a detailed description of the development of soft tissue and the functional anatomy of the larva. The duration of this developmental process depends on numerous factors, such as temperature, salinity, oxygen, and egg quality. Only a brief summary of larval development will be given here, as Eble in Chapter 2 and Carriker in Chapter 3 describe the anatomy and shell of the larva in more detail.

After fertilization occurs, meiosis results in the extrusion of a first and a second polar body, and cell division proceeds to produce a blastula rotating within the vitelline (egg) membrane after about 4 h (Galtsoff 1964). Shortly thereafter, a gastrula is formed, a girdle of cilia ("prototroch") develops, and the larva (trochophore) hatches from the egg and begins to swim. Because the larva has not yet started to grow, it remains about 50 μm in diameter.

Galtsoff (1964) observed the ontogeny of several batches of fertilized eggs that developed into trochophores in the laboratory at 22.5 to 24.5°C and 32.2 ppt salinity. The timing varied among samples, but ranged as follows (see text table, p. 362):

At 22 to 24°C, the trochophore persists for 24 to 48 h (Galtsoff 1964), before developing into the veliger (or D-shape) stage, which is protected by the larval shell. The terms prodissoconch I and prodissoconch II refer to the two sequential periods of bivalve

Stage of development	Time to reach each stage (range in min)
Fertilization membrane	5 to 25
First polar body	25 to 52
Second polar body	40 to 70
First cleavage	45 to 72
Second cleavage	52 to 120
Third cleavage	55 to 195
Morula stage	135
Rotating blastula	240 to 390
Trochophore	300 to 540

larval development that begin with shell production; the terms are also applied to the shell produced during these two periods. After settlement and metamorphosis, the shell that is laid down is called the dissoconch. These terms and stages of development in *C. virginica* are inter-related, as shown by Carriker in Chapter 3.

The veliger stage takes its name from the velum, or swimming organ (see Fig. 1, Newell and Langdon, Chapter 5). The velum, in common with the foot and byssus gland, is a larval structure that is resorbed after settlement (Baker and Mann 1994). It is a cup-shaped organ attached to the larval body by the peripheral velar membrane and the velar retractor muscles, and is formed by the enlargement of the prototroch. Principal cilia on the velum propel the larva through the water. The velum is very sensitive to disturbance, and can be withdrawn rapidly into the shell by velar retractor muscles, allowing the larva to sink. The velum also functions as the feeding organ, with the principal ciliary band generating the major feeding current (for further details on larval feeding see Newell and Langdon, Chapter 5)

Elston (1980) grouped oyster larval tissues into six functional organ systems: organs surrounding and delimiting the visceral cavity; the foot; the digestive system; the musculature; free cells of the visceral cavity; and rudimentary organs. The visceral cavity is a fluid-filled chamber delimited dorsally and laterally by the mantle, which secretes the shell, and which is limited ventrally by the velum. Within the visceral cavity are the digestive organs, musculature, and free cells. The foot begins development in the visceral cavity of the prodissoconch II stage, but as growth progresses it lies mostly in the mantle cavity. Paired left and right byssal ducts develop to connect the byssal gland with the posterior surface of the foot, opening to the mantle cavity. The digestive system is organized with the ciliated mouth positioned ventral-posteriorly, clearly distinguishable from the densely ciliated esophageal tube, which opens into an expandable stomach. There is an associated semi-rigid sac that is densely ciliated and in which the style is later formed. The digestive gland is H-shaped, and the intestine runs from the fecal groove of the stomach to the anus. The larval musculature includes the anterior and posterior adductor muscles and four paired groups of retractor muscles that are connected to the foot, mouth, and velum. The muscle bands of the velar retractors are cross-striated, indicating a capacity for rapid contraction. Various cells can be found in the visceral cavity, including phagocytic and non-phagocytic cells. Rudimentary demibranchs (gill plates) and presumptive vascular, nervous, and excretory tissue can be seen in the larval body cavities.

Extensive behavioral studies reviewed by Kennedy in Chapter 10 have shown that eastern oyster larvae respond to many physical factors, including light and salinity. Unfortunately, information on the structure and function of the larval sense organs is extremely limited due to the difficulty of performing electrophysiological studies on organisms as small as mollusc larvae. The foot of larval bivalves, including *O.edulis* (Waller 1981) and various species of scallop (reviewed by Cragg and Crisp 1991), contains a pair of statocysts that probably serve to detect motion (Cragg and Nott 1977). There is also a ciliated structure, termed the apical sense organ, in the center of the basal region of the velar cup, underlain by the cerebral ganglion (Galtsoff 1964; Elston 1980; Waller 1981). Its function is unknown, but Carriker (1986) speculated that it may control the selection and ingestion of food particles and play a role in the response of the veliger to suspended particles.

Fully grown bivalve larvae develop paired pigmented structures that have been termed "eyespots" because of their resemblance to eyes. Nelson (1926) reported that the eyespots of *C. virginica* appear to be sensitive to light, but more definitive research is required to determine if they are true photoreceptors. In the Pacific oyster, the eyespot on the right side appears first (Coon et al. 1990). The appearance of the eye-

spots has been taken by aquaculturists as evidence that the larvae are ready to settle and metamorphose, i.e., that they are "competent." Coon et al. (1990) found that behavioral competence in larval *C. gigas* could be induced experimentally after exposure to L-DOPA at the stage corresponding to the initiation of eyespot development, although behavioral competence did not seem to require the presence of fully developed eyespots (which generally appeared in larvae at 290 to 310 μm length). Some larvae with fully-developed eyespots exhibited no settlement behavior, whereas others that did show such behavior had no eyespots. Baker and Mann (1994) observed that eyespots gradually start to degenerate after metamorphosis. By the time the dissoconch postlarval stage is reached, the eyespots are represented by only a thin line of pigment.

FUTURE RESEARCH DIRECTION

Reproductive processes in the eastern oyster are generally well-understood, but there are uncertainties about some key points, as we have noted throughout the review. We believe that there is a great need for integrated field studies, preferably covering several consecutive years, of one or more populations of *C. virginica*, in which intraspecific variation (both between and within groups) in growth, reproductive output, and reproductive effort is measured. Such a study would provide much needed insights into the interrelations between energy storage, growth, and reproduction, and the way in which these processes respond to environmental change. A study of this nature is most likely to be feasible in an area free from disease.

A second major area of research on the reproductive ecology of bivalve molluscs that has received little attention in any species involves elucidating the relationship between exogenous and endogenous factors in synchronizing reproduction. Necessary insights may only be gained from multifactorial laboratory studies applying innovative endocrinological techniques for the analyses of hormonal cycles.

Overall, the accumulated body of research summarized here is based on a large number of small but focused studies, many of which are observational. What has been lacking is a conceptual framework in which to place these observations. We advocate the development of a quantitative framework in which the component reproductive descriptors (fecundity in relation to body size, size and spatial distribution of animals within a population, etc.) and processes (spawning events and their synchrony, diffusive and advective movement of gametes, half life of activity of sperm and eggs, dispersal of larvae, quantification of settlement as a kinetic event cued by a combination of boundary layer flow processes and chemical cues, etc.) are integrated. Such an exercise will serve to identify both the strengths and the weaknesses of the current literature.

Embedded within this framework must be a strong recognition of the unique nature of formerly pristine oyster populations, aggregated and arrayed in three dimensional reefs as stable biological, physical, and geological structures in estuarine and coastal ecosystems. This aggregated distribution is a strong indicator of the importance of spatial proximity and time dependent processes in oyster reproduction. Proximity influences chemical ecology in spawning, and in addition to absolute population size and size distribution within a population, dictates survival to the fertilized egg stage. Compared with spawning in an aggregated population of adult oysters, spawning in a uniformly distributed population will likely result in fewer fertilized eggs and consequent diminished synchrony of spawning, lowered fertilization efficiency associated with gametes of essentially no motile abilities, and limited half lives of activity. Most of these and other component reproductive processes can, in the first order, be approximated by simple mathematical relationships, at least as tools to develop testable hypotheses. The lack of tractable models to investigate population reproductive activity, exemplified in stock-recruit relationships for many other commercially exploited species, illustrates the need for a unified approach both for ecological research and for management needs.

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