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THE BIOLOGY OF GAMETE ACTIVATION AND FERTILIZATION IN *SICYONIA INGENTIS* (PENAEOIDEA); PRESENT KNOWLEDGE AND FUTURE DIRECTIONS

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W. H. Clark, Jr., T.-I. Chen, M. C. Pillai, K. Uhlinger, J. Shoffner-McGee and F. J. Griffin (1911) The biology of gamete activation and fertilization in *Sicyonia ingentis* (Penaeoidea); present knowledge and future directions. *Bull. Inst. Zool., Academia Sinica, Monograph* 16: 553-571. The marine shrimp, *Sicyonia ingentis*, is a close thelycum species. At mating sperm are transferred to female seminal receptacles where they undergo capacitation; a process required before sperm are competent to fertilize eggs. At spawning sperm and eggs are simultaneously released and commingled. The sperm are nonmotile unistellate cells. During sperm-egg interaction these cells bind to the egg vitelline envelope (VE), rapidly undergo exocytosis [the first phase of the acrosome reaction (AR)], and penetrate the VE. Sperm which have penetrated the VE become closely associated with a glycocalyx on the surface of the oolemma; the leading edge of the sperm is now an acrosomal granule, exposed during exocytosis. At this point a jelly precursor is released, from invaginations (crypts) in the eggs oolemma, which forms a jelly layer around the egg. Concomitant with jelly release is the resumption of meiotic maturation. After residing on the egg surface for 10-20 min the exocytosed sperm undergoes the second phase of the AR (filament formation). By approximately 30 min sperm incorporation into the egg occurs and the egg undergoes a cortical reaction. This reaction is characterized by the elevation of the glycocalyx from the surface of the egg and the formation of a hatching (fertilization) envelope. The events of activation described above (*i. e.* jelly release, resumption of meiosis and hatching envelope formation) are independent of a sperm's presence. The physiological characterization of the above events, as well as the development of techniques making such characterizations possible, are reviewed. In addition, a method for *in vitro* fertilization is presented.

Key words: *Sicyonia ingentis*, Fertilization, Gamete activation.

Our laboratory, over the last decade and a half, has directed a great part of it's research efforts toward unraveling the mechanisms by which fertilization and early development are achieved in penaeoidean shrimp. At the initiation of our work there was virtually nothing in the contemporary literature concerning gamete physiology or the fertilization biology of the Penaeoidea. This was in great part due to the fact that penaeoideans utilize fertilization strategies seemingly quite aberrant when compared to accepted "models" developed with other systems (see Longo, 1987); thus, techniques that had been developed for the experimental manipulation of gametes in these other systems were not readily transferable to the penaeoideans. Our need was to find a penaeoidean species that was readily available, easily maintained, and one whose reproductive success was not adversely affected by handling and maintenance in the laboratory. The animal of choice in our laboratory and one that we believe has developed into a model system for penaeoidean fertilization and development studies is *Sicyonia ingentis*.

Recent papers have discussed in detail our work on the mechanisms through which *S. ingentis* sperm become activated to undergo an acrosome reaction (Clark and Griffin, 1988; Griffin and Clark, 1990) and eggs become activated to begin development (Clark and Pillai, 1990; Pillai and Clark, 1987, 1988, 1990; Pillai *et al.*, 1990). In the present paper we review these works in the context of the general reproductive biology of *S. ingentis*. In addition, discussed in the context of our ability to reproductively manipulate this animal, we present data on the relationship between female molt stage and mating, describe the mechanisms by which sperm and eggs are brought together at spawning, and present the results of *in vitro* fertilization studies. Lastly, we briefly present our perspectives on future research directions.

GENERAL REPRODUCTIVE BIOLOGY

Sicyonia ingentis, a penaeoidean shrimp belonging to the family Sicyonidae, is indigenous to the eastern Pacific ocean. The geographic range of *S. ingentis* extends from Monterey Bay, California (USA) in the north, to Isla Maria Madre,

Gulf of California (Mexico) in the south (Perez-Farfante, 1985). Although found in tropical waters (*e. g.*, the Gulf of California), it also survives well and reproduces in temperate waters as evidenced by the depths at which it is found off of the southern California (USA) coast, where temperatures may be as low as 12-14°C. Within the northern range (up to latitude 34°N) the female reproductive season extends from mid to late June through late October; males appear to be reproductive throughout the year although a decrease in the number of sperm produced are seen in the months of November through January (Anderson *et al.*, 1984, 1985). Mating between males and females appears to occur from February through at least September or October, as evidenced by collections of sperm bearing females from wild caught populations (unpublished data). Males transfer sperm masses into the seminal receptacles of females which are in pocketings of exoskeletal arthrodial membranes and are located on the ventral cephalothorax between the fourth and fifth pairs of walking legs (Perez-Farfante, 1985; Suverkropp, 1990). Females retain sperm from matings until they either spawn or undergo ecdysis. Since seminal receptacles are exoskeletal they and any sperm within them are shed with the old exoskeleton at ecdysis. Should a female molt after mating, but prior to having completed ovarian maturation and spawning,

Table 1
Postmolt age of females in laboratory matings

Postmolt age-start	*N*	Postmolt age-mated		Females with sperm	Days with male at mate
		Mean	Range		
0	8	5.6	3-10	6	5.6
1	5	2.8	2-5	1	1.8
2	11	4.2	2-10	8	2.2
3	4	6.2	3-15	1	3.2
4	2	4.0	—	1	0
6	1	6.0	—	0	0
Overall	31	4.6	2-15	17	2.1

Isolated female *Sicyonia ingentis*, at specified days postmolt (Postmolt age-start), were placed with males (2 males/female) and monitored by video for precopulatory behavior and mating. After mating was observed, the seminal receptacles of females were excised and examined for the presence of sperm (Female with sperm).

she will remate. Under laboratory conditions such matings do not take place immediately (Table 1). Females (ranging in post-molt age of 0-6 days) were placed with males and monitored using time lapse video. In 31 trials, the average post-molt age was 4.6 days; significantly, no female was mated at less than 2 days post-molt and all females (n=3) that were≥10 days post-molt were successfully mated, that is they had received sperm. This datum suggests that either males and females require a period of familiarization with each other or that females are not mated in the fresh post-molt condition. Regardless of which condition is operative, our experiments demonstrate that *S. ingentis* is capable of mating and transferring sperm when females are several days post-molt, that is when they are no longer in the "soft" condition.

Female *S. ingentis* exhibits a characteristic pre-spawning behavior that correlates with the occurrence of ovulation and signals that spawning is eminent (Pillai *et al.*, 1988). They repeatedly rise into the water column and passively drop back down to the substratum, and eventually remain in the water column with the aid of their pleopods until after they have spawned (Pillai *et al.*, 1988). *S. ingentis* also exhibits a predictable behavior just prior to and associated with the act of spawing itself. This includes: 1) a rapid increase in pleopod beat frequency and, at the same time, a decrease in the velocity or cessation of forward movement in the water; 2) a ventrally directed bending or curving of the abdomen, which may be responsible for the slowed forward movement; and, 3) a dorso-anterio extension and left/right alternate beating of the fourth pereopods. Within seconds of initiation, while continuing this behavior, the female release eggs from her paired ovopores (at the bases of the third pair of walking legs). The first two behaviors undoubtedly aid in the mixing of gametes at their release insuring that sperm-egg contact occurs (Pillai *et al.*, 1988); sperm-egg mixing is essential since *S. ingentis* sperm are non-motile (Kleve *et al.*, 1980).

It had been suggested previously that pereopod movement during spawning (the last behavior described above) functions in the release of stored sperm (Hudinaga, 1942; Motoh, 1981). Since the spawning behaviors of *P. japonicus*

(as described by Hudinaga, 1942) and *S. ingentis* (as reported by us) were similar and since our observations suggested that neither egg release nor the mixing of released gametes were controlled by pereopod movements, we hypothesized that the mechanism for sperm release by

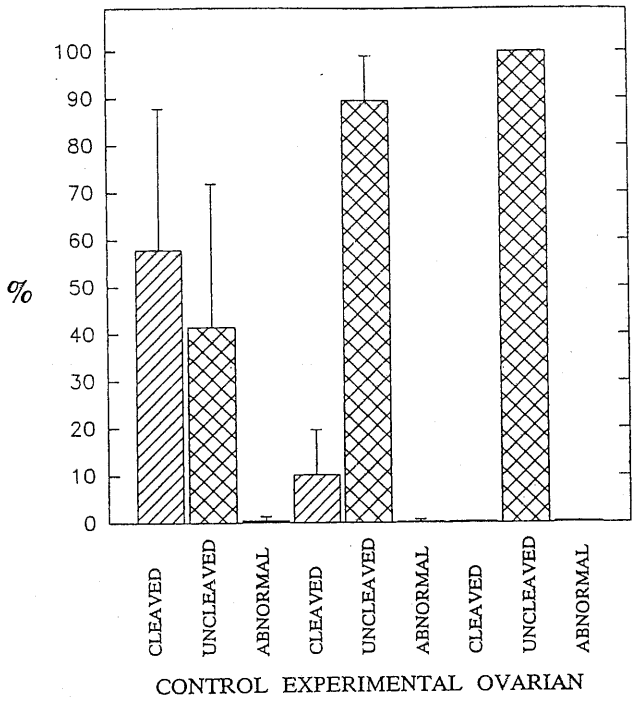

Fig. 1. The effect of pereopod restraint on fertilization in *Sicyonia ingentis*. Female *S. ingentis* were induced to undergo ovulation and spawning as described by Pillai *et al.* (1989). If a female exhibited normal spawning behavior (see Pillai *et al.*, 1988) she was placed on a beaker containing sea water and allowed to spawn for 15 sec. She was then removed from the beaker, and after her pereopods had been restrained and her ventral cephalothorax washed with sea water, she was placed on another beaker and allowed to spawn (with pereopods restrained) for 15 sec. Subsequently, eggs were dissected from the ovary and placed in a third beaker of sea water. All samples were incubated at 20-22°C for 90 min and scored for percentage of eggs that had undergone normal cleavage (which correlates with fertilization; Pillai and Clark, 1987). In each experiment (n=15) 300 eggs were scored from a normal spawn (CONTROL), 300 from a pereopod restrained spawn (EXPERIMENTAL), 300 that had been removed from the ovary and incubated in sea water (OVARIAN; a negative control in which there should be no fertilization). Differences in percentages of cleavages between CONTROL and EXPERIMENTAL, and percentages uncleaved between the same treatments were significant (t=5.874; $p<$ 0.001 and t=5.856; $p<0.001$ respectively).

S. ingentis females was associated with the rhythmic pereopod movement we had observed during spawning. To test this we conducted experiments in which pereopod movements were restricted during spawning (Fig. 1). When females were allowed to exhibit normal spawning behavior (positive controls), the fertilization rate (indicated by percentage of eggs exhibiting normal cleavage) was 58%. When the fourth pair of pereopods were physically restricted during spawning, the percentage fertilization dropped to 10%, comparable to that of eggs removed from ovaries and placed in sea water (negative controls; 0%). As expected similar results were obtained when numbers of sperm bound to eggs in pereopod restriction spawns and normal spawns were compared (Shoffner, 1989).

PRE-ACTIVATIONAL GAMETES

Sicyonia ingentis eggs, depicted in Fig. 2A, are isolecithal, approximately 250 μm in diameter, and arrested in first meiotic-metaphase. They are surrounded by a 0.1 μm thick extracellular matrix (ECM), the vitelline envelope. They lack an enveloping jelly layer prior to activation (spawning); jelly precursor, in the form of tightly packaged "bottle-brush" structures is present. This precursor is packed in cortical crypts, regularly spaced regions where the oolemma is recessed into the cortex of the egg (Figs. 2A and 2B). Over non-crypt regions of the egg surface, between the vitelline envelope and the oolemma, there is a granular material that will give rise to an ECM termed the surface coat (Fig. 2B). Lastly, ring-shaped structures, contained within cisternae in the cortical ooplasm will contribute to the formation of a hatching envelope.

Sperm, prior to activation, can be divided into three regions, a posterior main body, a central cap and a spike that is directed anteriorly (Fig. 3A). The sperm can also be subdivided into three functional regions. The first is the acrosomal vesicle in the anterior region of the sperm that contains the spike, anterior granule and membrane pouches (Fig. 3B); these are exposed after exocytosis of the acrosomal vesicle and function in gamete binding and fusion. The second region is the subacrosome which is separated from the acrosomal vesicle by the inner acrosomal

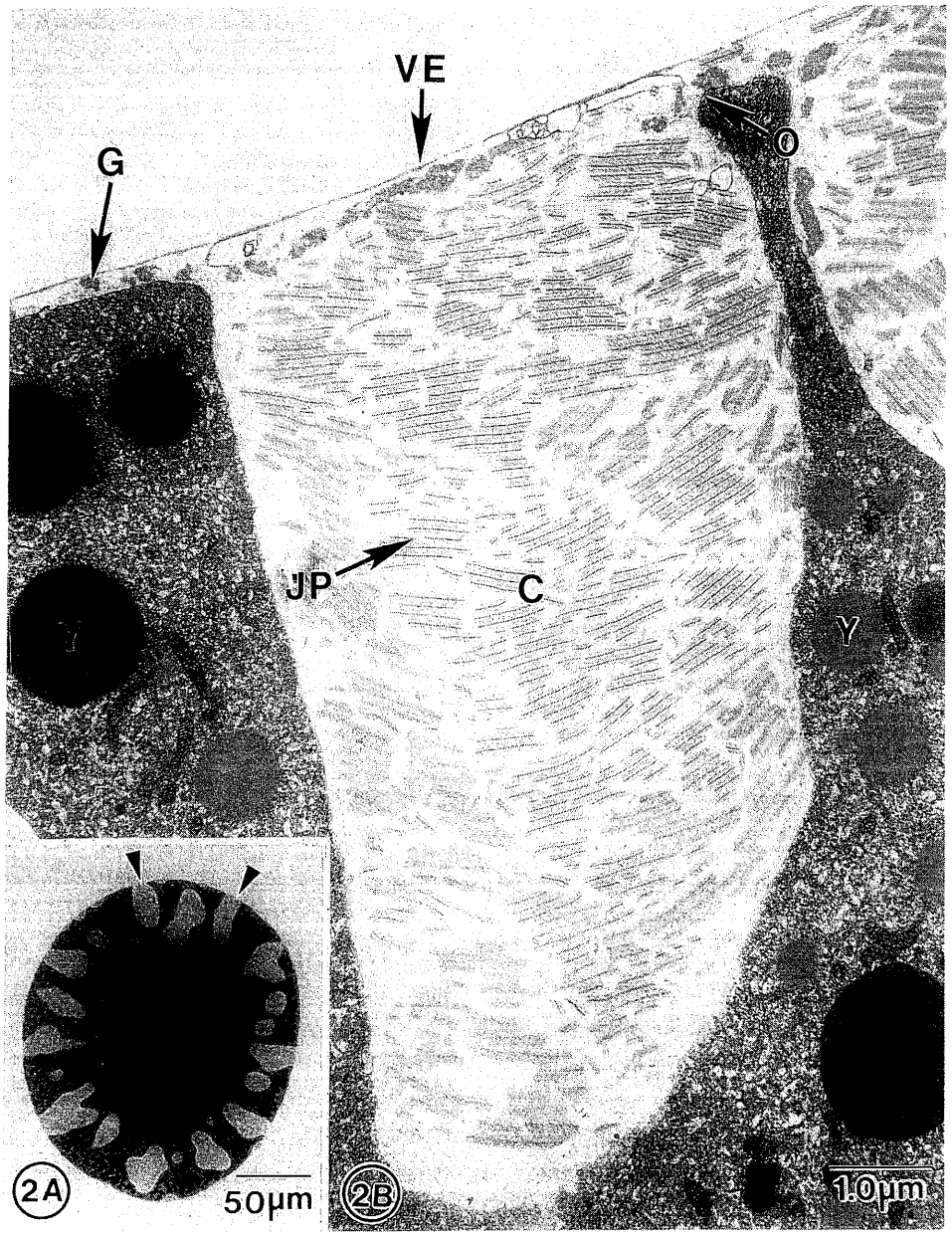

Fig. 2. An *Sicyonia ingentis* egg spawned into fixative (time "0"). 2A is a light micrograph of a sectioned egg depicting the extracellular cortical crypts (arrowheads) containing jelly precursor. 2B is a transmission electron micrograph depicting jelly precursor (JP) constrained withins a cortical crypt (C) that is delineated by the invaginated oolemma (O) and overlying vitelline envelope (VE). Granular material (G) can be seen in non-crypt regions situated between the VE and O; Y=yolk. Modified from Clark *et al.*, 1990.

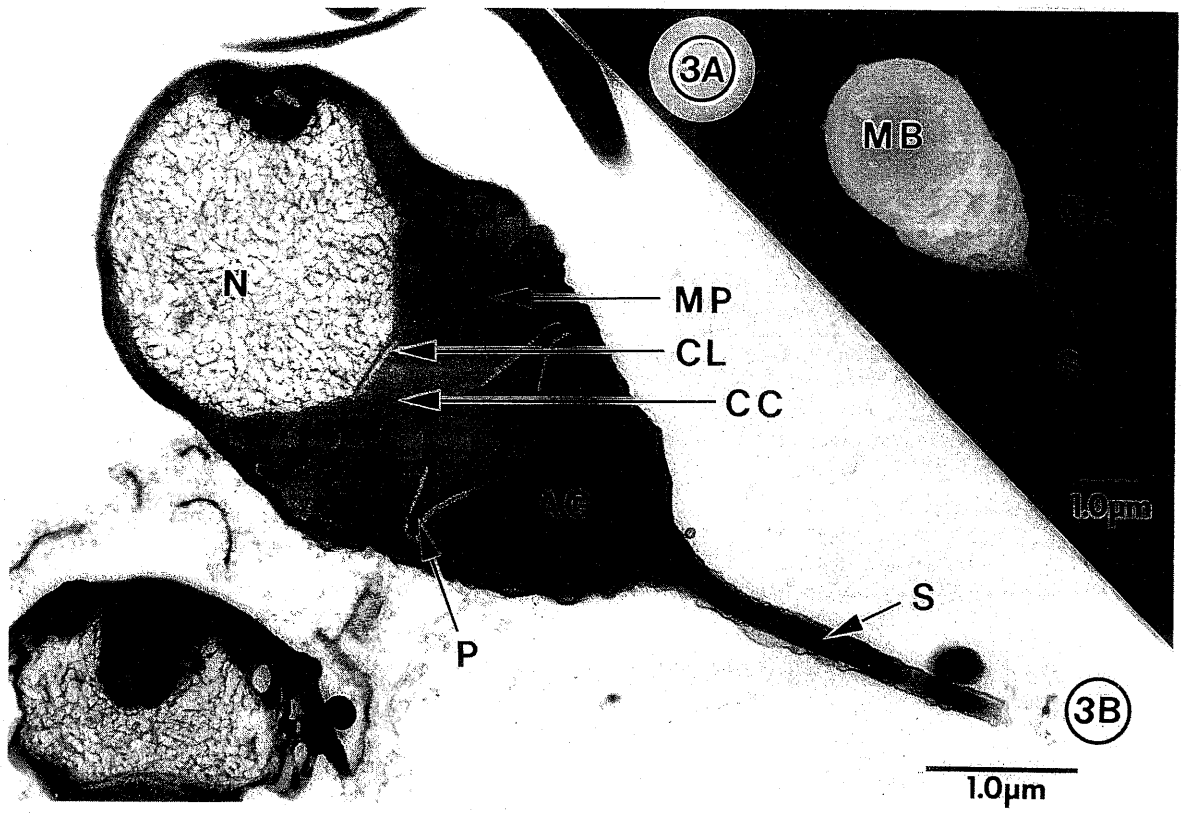

Fig. 3. An unreacted *Sicyonia ingentis* sperm. A scanning electron micrograph (3A) depicting the posterior main body (MB), the central cap region (CA), and the anterior spike (S). In a transmission electron micrograph (3B) the nucleus is visible within the posterior portion of the sperm. The acrosomal vesicle contains the membrane pouches (MP), the anterior granule (AG), and the anterior spike (S). The crystalline lattice (CL), cylindrical core (CC), and the petals (P) constitute the subacrosome.

membrane, is centrally situated in the sperm, and is the structure from which the acrosomal filament is formed (Fig. 3B). The third region is the posteriorly positioned main body which houses the uncondensed nucleus (see Clark *et al.*, 1981; Clark and Griffin, 1988).

SPERM CAPACITATION

Not only are sperm transferred to and stored by the female prior to contacting and fertilizing an egg, they are required to do so, that is, sperm removed from males (either the vas deferens or terminal ampoule) are not competent to fertilize an egg. In an attempt to understand the changes that bestow competency on sperm to acrosome react, we undertook studies of both competent (capacitated) and non-competent

(uncapacitated) sperm. The early processes of spermiogenesis in *S. ingentis* have been previously described (see Shigekawa and Clark, 1986). Testicular spermiogenesis was categorized into seven stages beginning after the completion of meiosis and culminating with subacrosomal development. The stage 7 spermatid, however, is neither capable of fertilizing an egg nor has it completed spermiogenesis at it leaves the testes. Such sperm possess incomplete subacrosomal regions and acrosomal vesicles, and lack an anterior spike. Terminal spermiogenesis (*i.e.*, completion of spermiogenesis) takes place during sperm transport through the vas deferens of the male (Shigekawa and Clark, 1986; Almeida and Clark, unpublished data). In the vas deferens, spermatids undergo continued development of the subacrosome and elongation of the acrosome's anterior spike. In addition seminal fluid constituents are added and sperm masses are packaged in an acellular envelope.

Although these cells superficially appear to be mature, there are important morphological and behavioral differences between them and sperm that have been tranferred to and have resided in the female's seminal receptacles. Morphologically changes are observed in both the subacrosome and acrosomal vesicle (Wikramanayake *et al.*, 1988). Such differences, as well as possible others that are as yet undescribed, manifest themselves in critical behavioral differences. Although capable of recognizing and binding to eggs (Wikramanayake and Clark, unpublished data), sperm removed from males will not activate (*e.g.*, undergo an acrosome reaction) in response to egg contact or contact with egg derived inducers and thus are incapable of fertilizing an egg (Wikramanayake, 1988).

SPERM-EGG INTERACTION

These atypical cells (sperm and eggs), not so surprisingly, also appear to undergo rather atypical activational events. The following summarizes our understanding of the events surrounding sperm-egg interaction and fertilization in *S. ingentis* (see Fig. 4). Ovulation in *S. ingentis* releases eggs from their enveloping follicular layer, but does not release such eggs from quiescence or arrest (Fig. 4A). Upon release by the female at spawning *S. ingentis* eggs contact sea water which initiates: 1) the resumption and completion of meiosis (Pillai and

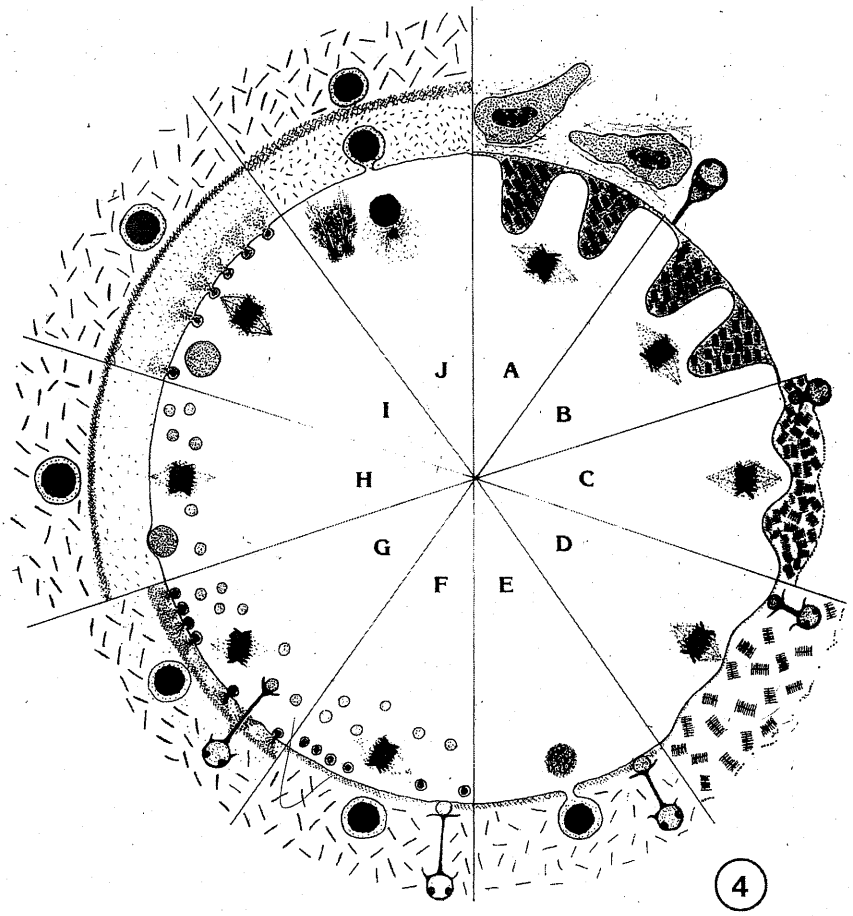

Fig. 4. Depiction of the observable events, in chronological order, of gamete activation and fertilization in *Sicyonia ingentis*. At ovulation oocytes are arrested in first meiotic metaphase, enveloped in an acellular vitelline envelope, and possess extracellular crypts that contain jelly precursor (JP) (Fig. 4A). At spawning sperm bind, *via* the tip of their appendage, to the egg's vitelline envelope (Fig. 4B). Within seconds sperm undergo acrosomal exocytosis, penetrate the vitelline envelope, and bind to the egg surface glycocalyx; egg activation is evidenced by extrusion of JP, elevation of the vitelline envelope, and release of the egg from meiotic arrest (Fig. 4C). Approximately 10-20 min after sperm-egg contact, JP undergoes transition to an enveloping jelly layer, sperm begins generation of an acrosomal filament, and the egg extrudes the first polar body (Figs. 4D & 4E). Prior to sperm-egg fusion (which occurs *circa* 30 min) two populations of cortical vesicles become evident in the egg cortex (Fig. 4F). Shortly after sperm-egg fusion the first population of cortical vesicles exocytose, coalescing with and lifting glycocalyx from egg surface; marking the begining of hatching envelope formation (Fig. 4G). Male pronucleus becomes evident and remains in the egg cortex until completion of meiotic maturation (Fig. 4H). Second population of cortical vesicles exocytose; their contents fuse with and complete formation of hatching envelope (Figs. 4I & 4J). Second polar body is released and both male and female pronuclei are observed prior to syngamy (Fig. 4J). Modified from Clark *et al.*, 1990.

Clark, 1987); and 2) the dramatic alteration and reorganization of the egg's ECMs (Pillai *et al.*, 1990). It is also at this time that sperm are released from seminal receptacles and are brought into contact with eggs by the female. Sperm that contact eggs bind to the vitelline envelope with the tips of their spikes (Fig. 4B) and almost immediately undergo acrosomal exocytosis (Clark *et al.*, 1981; Clark *et al.*, 1984). As a result of having undergone acrosomal exocytosis, sperm are seen to have penetrated the vitelline envelope and become bound to the surface of an egg (Fig. 4C). In response to an egg contacting sea water, jelly precursor ("bottle-brush" structures) begins to exit from the extracellular crypts in the egg, lifting the vitelline envelope and any sperm that have failed to penetrate it from the surface of the egg (Fig. 4C). As the jelly precursor continues to emerge, the "bottle-brush" structures begin to slowly give way to an expanding flocculent matrix, such that the contents of neighboring crypts meet, creating a corona of jelly and jelly precursor around the egg (Clark *et al.*, 1990) (Fig. 4D). By approximately 15 min post-spawning, a flocculent jelly layer is evident surrounding the egg (Fig. 4E). A fibrous surface coat, situated between the oolemma and the jelly layer is also evident by this time. Thus, within the first 15-20 min after release, one ECM (the vitelline envelope) is lost and two new ECMs (the surface coat and jelly layer) are formed (Pillai *et al.*, 1990). It is also during this first 15-20 min that the resumption of meiosis occurs, as evidenced by the extrusion of the first polar body (Pillai and Clark, 1987) (Fig. 4E).

The sperm that have remained on the surface of the egg since undergoing acrosomal exocytosis at first sperm-egg contact, complete the acrosome reaction by generating an acrosomal filament approximately 10-20 min post spawning (see Figs. 4D and 4E). This filament requires 3-5 min to form once extension is initiated, is approximately 10 μm long, and terminates anteriorly in 12-15 laterally radiating petal like extensions (Griffin *et al.*, 1988). Within the egg, during the time span of 15-30 min post-spawning, two distinct types of cortical vesicles appear in the egg cortex (Fig. 4F). The first, dense vesicles, are derived from Golgi bodies after egg activation (Pillai and Clark, 1988, 1990). The second, the ring vesicles, are formed from the cisternal elements

that were present prior to activation (Pillai and Clark, 1988, 1990).

Although the exact time and circumstances of sperm-egg fusion have not yet been established for any penaeoidean species, it appears likely that gamete fusion in *S. ingentis* occurs *circa* 30 min post-spawning (at 20°C) and may involve a rather extensive egg membrane disillusion in contrast to a text book fusion (Chen and Clark, unpublished data). We do know that confluence between the sperm and the egg must be established by 30-35 min post-spawning. It is at this time that formation of the hatching envelope is initiated by the exocytosis of the first population of cortical vesicles (Pillai and Clark, 1988, 1990) (Fig. 4G). The exudate combines with the surface coat to form the "thin" hatching envelope, which elevates from the surface of the egg (Fig. 4H). Thus, at this point in time (35 min post-spawning) sperm that have not fused with or become confluent with an egg will be denied further access to the oolemma. By 40 min the second population of cortical vesicles undergoes exocytosis (Fig. 4I), adding a second layer to and completing the formation of the hatching envelope (Pillai and Clark, 1988, 1990) (Fig. 4J). Subsequent to the completion of the hatching envelope the extrusion of the second polar body is observed signaling the completion of meiosis (Pillai and Clark, 1987) (Fig. 4J).

PHYSIOLOGY AND CONTROL OF GAMETE INTERACTION

All of the activational events described aboved for *S. ingentis* eggs are elicited by contact with the ionic milieu of sea water. Extracellular Mg^{++} is required for the initiation and completion of all observable events (Pillai and Clark, 1987). In Mg^{++}-free sea water, jelly precursor is released from the extracellular crypts of *S. ingentis* eggs, but it's transformation into a flocculent jelly layer is blocked (Pillai and Clark, 1987); in other penaeoideans, *Penaeus aztecus* for example, even release of jelly precursor from crypts is blocked in the absence of Mg^{++} (Lynn and Clark, 1987). Additionall, resumption of meiotic maturation and hatching envelope formation are blocked by such deletions. Curiously, the extracellular ionic requirements of fertilized and unfertilized eggs appear to differ. In the presence of fertilizing sperm, there is no

extracellular Ca^{++} requirement, however, unfertilized eggs will activate only if extracellular Ca^{++} (in addition to Mg^{++}) is present (Pillai and Clark, 1987).

It is the sperm's contact with the egg that sets in motion the ionic changes that elicit the acrosome reaction (Clark and Griffin, 1988). Our *in vitro* studies of the acrosome reaction have allowed the dissection of the mechanisms of both induction and ionic control of the phases (acrosomal exocytosis and filament formation) of the acrosome reaction. Prior to activation, *S. ingentis* sperm taken from the seminal receptacles of females possess an unusually high intracellular pH (pH_i) of 8.47 and after undergoing both phases of the acrosome reaction (exocytosis of the acrosomal vesicle and generation of the acrosomal filament) exhibit a significantly lower pH_i of 7.81-8.01 (Griffin *et al.*, 1987). Although the presence of extracellular Ca^{++} is required for the induction of the first phase of the acrosome reaction, loss of the spike and exocytosis of the acrosomal vesicle (Clark *et al.*, 1981), a transcellular flux of Ca^{++} does not appear to be required (Lindsay and Clark, unpublished data). Evidence utilizing ionophores has suggested that the pH_i drop is required for filament formation and not acrosomal exocytosis (Griffin *et al.*, 1987) although the pH_i shift occurs well before filament formation and of itself is not sufficient to elicit filament elongation (Lindsay and Clark, unpublished data). Regarding induction, we have demonstrated: 1) the two phases of the acrosome reaction are induced by separate egg derived inducing activities; 2) the inducing activities are isolated with or within a high molecular weight ($>1\times10^6$ D) complex that contains among other components, carbohydrate moieties and trypsin-like proteolytic activity; 3) the activity of an egg-derived trypsin-like protease is required for induction of the second phase of the acrosome reaction and filament formation (Griffin and Clark, 1990).

IN VITRO FERTILIZATION

The idiosyncracies of penaeoidean gamete interaction, activation and fertilization do not make these cells conductive to the types of manipulations commonly utilized for *in vitro* fertilizations of aquatic animals. These include: 1) The release of fertilizing sperm and eggs by the female at spawning; to effect an *in vitro* fertilization, techniques must be

used to prevent a female from releasing stored sperm or utilize females that have not mated. 2) Sperm must undergo capacitation before gaining the competence to activate; this means that sperm obtained from males cannot be directly used for *in vitro* fertilizations. 3) The sperm are non-motile; thus eggs and sperm must be physically mixed in an *in vitro* situation. 4) Sperm have a very short period of time within which they must bind to and penetrate the vitelline envelope of an egg; therefore unactivated eggs must be quickly added to and mixed with sperm, and not *visa versa* as is possible in other systems. These "obstacles" to penaeoidean *in vitro* fertilization have been dealt with using the following techniques.

To prevent a female from applying her stored sperm to eggs at spawning, eggs were obtained by: 1) dissection of the ovary of an ovulated female (see Pillai and Clark, 1987); or 2) collection from a spawning female whose pereopods were immobilized. This prevents the spawning female from releasing her stored sperm, as discussed previously, and thus does not allow her to fertilize her spawn. Capacitated sperm to be used for *in vitro* fertilizations were collected and pooled from seminal receptacles of non-spawning females as described by Griffin *et al.* (1987) and utilized 1) fresh, within 60 min of isolation; or 2) after cryopreservation and storage at liquid nitrogen temperatures (Anchordoguy *et al.*, 1988).

We overcame problems associated with sperm non-motility and the fact that eggs activate upon contact with sea water and rapidly lose ability to interact with unreacted sperm, by the following. Isolated sperm were suspended in sea water at a concentration of 10^7 cells per ml. Unactivated eggs were then added to the sperm suspension and physically mixed by gentle pipetting for about 5 min. To ensure that we were indeed successfully *in vitro* fertilizing *S. ingentis*, these treatments were compared to both positive and negative controls. Experiments were conducted as follows. Gravid wild-caught females were light cycled to elicit ovulation (Pillai *et al.*, 1988). Ovulated females were then induced to spawn (probe spawned) and once a female exhibited a normal spawning behavior (consisting of an anterior extension of the pereopods in conjunction with alternate jerking of each of the fourth pereopods and continuous beating of the pleopods;

(see Pillai *et al.*, 1988), they were first moved to a fresh beaker containing sea water and allowed to spawn normally for approximately 15 sec without restrictions (positive control). The female was then removed from the beaker, her pereopods were immobilized, the ventral cephalothorax was washed quickly with sea water to remove any adhering previously spawned or released sperm and/or eggs. Then, with pereopods immobilized, females were placed on a beaker of sea water only and allowed to release eggs for 15 sec (negative control), and then transferred to a beaker containing previously isolated fresh or cryopreserved sperm (experimental). Eggs were subsequently transferred to 250 ml finger bowls and monitored until positive control eggs were beginning to undergo second cleavage (*circa* 120 min post-fertilization); since cleavage is relatively synchronous in *S. ingentis*, this ensured that we had allowed enough time for first cleavage to occur (*circa* 90 min) in all samples (see Pillai *et al.*, 1988). An egg was deemed fertilized if an equal and complete first cleavage occurred; *S. ingentis* eggs that are unfertilized either do not cleave or undergo an aberrant, pseudocleavage (Pillai and Clark, 1987).

The results depicted in Table 2 clearly demonstrate that our technique for *in vitro* fertilization is successful. The mean fertilization rate of all unrestricted probe spawns was 57.8%; we recently reported a normal fertilization rate of 65.5%

Table 2
In vitro fertilization in *Sicyonia ingentis*

Treatment	*N**	Mean (%)	Range (%)
In vitro-fresh sperm	23	59.9	23.0–93.5
In vitro-cryopreserved sperm	4	62.3	32.0–82.0
Non-fertilized-pereopod	15	17.8	0–68.0
Non-fertilized-ovarian	8	0.9	0– 5.0
Normal spawns	22	57.8	7.5–98.0

* *N*=number of experiments. All experiments (23) included an *in vitro* fertilization utilizing freshly isolated sperm (*In vitro*-fresh sperm) and a negative control that was either a pereopod restricted spawn (Non-fertilized-pereopod) or eggs removed directly from a dissected ovary (Non-fertilized-ovarian). Four of the experiments also included a trial utilizing previously cryopreserved sperm (*In vitro*-cryopreserved sperm). All but one experiment also included a trial in which the pereopods were not restricted, thus allowing the female to fertilize her spawn with her own stored sperm (Normal spawns).

from animals that were probed to spawn and 75.5% from animals that were allowed to spawn naturally (Pillai *et al.*, 1988). The reduced percentage obtained here for probe spawns is due to the fact that our current figure includes 4 spawns in which normal spawning behavior did not occur and fertilization was 12% or less; these were included in the data set because both negative controls and experimental samples were comparable to those in other experiments. Spawns in which pereopods were restricted, thus preventing sperm release by the female, had a mean fertilization rate of 17.8% (Table 2). As would be expected, eggs removed from dissected ovaries (and thus not allowed contact with sperm) averaged a much lower 0.9% fertilization. The overall *in vitro* fertilization rate using freshly collected sperm was 59.9% (Table 2). The *in vitro* fertilization rate for eggs obtained by pereopod restriction (63.5%) was slightly higher than that for ovarian eggs (53.1%); this no doubt reflects the differences in background fertilization rates between the two egg aquisition methods, 0.9% fertilization in ovarian eggs as opposed to 17.8% in pereopod restricted eggs.

In four of the experiments eggs were mixed *in vitro* with previously cryopreserved sperm (Table 2). Cryopreserved sperm yielded a mean fertilization rate of 62.3%, slightly higher than, but comparable to the overall *in vitro* rate of 59.9% that was obtained with freshly collected sperm.

CONCLUSION

A relatively high degree of control has been obtained over the reproductive cycle of *S. ingentis*. This control has provided the tools necessary to carefully dissect the events of gamete activation and interaction in a penaeoidean shrimp. As discussed in this report several of the above events have been characterized (*e.g.*, the sperm acrosome reaction, egg activation, etc.). It needs to be stressed, however, that many of the events leading to zygote formation remain unknown. Our laboratory continues work with this system and is actively pursuing two main avenues of research.

First, the biochemical nature of binding and activational molecules [required for sperm-egg recognition, induction of the first phase of the sperm acrosome reaction (exocytosis), and post-exocytotic sperm-egg binding] remain unknown. This

work has been difficult due to the lack of availability of competent sperm. That is, capacitated sperm must be obtained from females and are available in relatively small numbers. Thus, an understanding of the capacitation process and techniques for *in vitro* capacitation are very important for the success of future research. Although we have established that sperm undergo a capacitation process after transfer to the female, the mechanisms (biochemical and/or ionic) by which capacitation occurs need to be elucidated. We have initiated studies to catalogue and identify capacitation associated changes in seminal fluid. Our goal in these studies is the identification and eventual isolation of a capacitation factor(s) that we suspect is either of female origin or although present in the male is activated after transfer to the female. In addition, the types of changes that we have observed in sperm during capacitation (Wikramanayake *et al.*, 1988) would suggest that these cells are actively metabolizing. Once capacitated, however, sperm possess very high ametabolic intracellular pHs (Griffin *et al.*, 1987). As such, we are also investigating the role of pH (as well as other ions) in the capacitation process.

The second area of research which we are pursuing is related to some very interesting questions that have arisen concerning the activation of eggs. It is unclear at this point whether the activations of fertilized and unfertilized eggs are equivalent. We do know that although unfertilized eggs appear to activate, they are not developmentally competent (Pillai and Clark, 1987). Thus, two questions immediately arise: 1) do unfertilized eggs in fact undergo an activation identical to fertilized eggs? and if so, 2) what renders such eggs developmentally incompetent? These studies will of necessity involve better defining the sequences and timing of activational changes and elucidatation of the contribution by the fertilizing sperm to developmental competence of the egg. For example, do sperm contribute a functional centrosome to an egg at fertilization?

In conclusion, we believe that *S. ingentis* has become an important model system with which to understand the basic mechanisms of reproductive biology in the penaeoideans. The cellular events of reproduction in this group of organisms has remained virtually unstudied even though these animals represent one of the most important wild and

culture fisheries in the would.

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