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[REVIEW]

The Urodele Egg-Coat as the Apparatus Adapted for the Internal Fertilization

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ABSTRACT—Fertilization is a significant event for reproducing offspring. It is achieved under a species-specific environment, which influences the conditions to assure the successful fertilization in some cases. Several studies about the basic mechanism of fertilization suggest that the fertilization mechanism is modified among species to be suited for the fertilization environment.

In amphibians, many anurans undergo external fertilization while most urodeles do internal fertilization. An amphibian egg is surrounded by egg-coats, which are composed of vitelline envelope and layered egg-jelly. They are significant as fields for the sperm-egg interaction at fertilization. The fertilization processes that take place in the egg-coats are supposed to be easily influenced by the fertilization environment, because they, especially egg-jelly, are exposed to the surroundings at fertilization. In the present article, we describe the fertilization system equipped in newt egg-coats. Newt sperm are stored in spermatheca that exists in cloaca of a female and directly inseminated on the surface of egg-jelly. Sperm motility and acrosome reaction are induced in the outermost portion of the egg-jelly. Motion of the moving sperm becomes vigorous in the egg-jelly and sperm are guided to vitelline envelope by the aid of egg-jelly structure. Most of the sperm passing through the egg-jelly, as the result, has been induced acrosome reaction and those sperm can bind to the vitelline envelope to contribute to the successful fertilization. This fertilization system has a distinct feature from the known system in species undergoing external fertilization. The feature of the system in the newt egg-jelly is discussed with the view to achieving the successful fertilization in the internal environment.

Key words: internal fertilization, newt, egg coat, egg-jelly, sperm-egg interaction

In amphibians, there are huge numbers of species living in any one place, in water or on land, where they can adapt to the surroundings. Fertilization, however, depends on the aqueous conditions, and it can be achieved in various modes (Duellman and Trueb, 1994). Amphibian fertilization can be divided into twenty-nine types (Duellman and Trueb, 1994), which may be one of the major factors in producing the diversity of amphibian species. There are also three different types of fertilization sites for amphibians. In many anurans and an ancient group of urodeles, eggs are externally fertilized with sperm inseminated in water, whereas most urodeles undergo internal fertilization (Wake and Dickie, 1998). Urodele females pick up the spermatophore ejaculated from males in water. Sperm packed in the spermatophore are released in the cloaca and stored in the spermatheca, which is a sperm reservoir at the exit of the oviduct (Greven, 1998; Sever, 2002). After a long storage period, they are directly inseminated on the eggs in the cloaca, and fertilization is thought to be completed before the inseminated eggs are spawned into water. In some species, sperm stored in spermatheca go into the oviduct and fertilize the egg (Greven, 1998). The fertilized eggs begin to develop in the oviduct, and embryogenesis is completed there. In those viviparous species, some carbohydrates are secreted in the posteriormost portion of the oviduct, uterus, to provide for the embryo (Greven, 1998). This is a specific event for species undergoing fertilization in the viviparous mode, suggesting that some of the oviductal secretions are modified for species-specific fertilization mode. Thus, though the basic mechanism for fertilization is common among amphibians, it may be modified to fit with the organism's surroundings. Amphibian fertilization system has been studied well in

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anurans undergoing external fertilization. In this paper, as a representative of a species undergoing internal fertilization, we have demonstrated the newt fertilization system, which includes the egg-coats, egg-jelly and vitelline envelope, that are exposed to and may be severely affected by the surrounding environment.

Egg-jelly

Amphibian egg-jelly is essential to successful fertilization and development. The sperm-egg interactions occur in the egg-jelly at fertilization (Picheral, 1977; Campanella and Gabbiani, 1979; Campanella *et al*., 1997; Al-Anzi and Chandler, 1998; Olson and Chandler, 1999; Nakai *et al*., 1999; Ukita *et al*., 1999; Onitake *et al*., 2000; Sasaki *et al*., 2002), and the egg-jelly prevents excess sperm from reaching the egg-surface after the egg is spawned into water (MacLaughlin and Humphries, 1978; Matsuda and Onitake, 1984a). The surface of egg-jelly is often sticky and makes it possible for the spawned eggs to adhere to the substrates. Furthermore, the egg-jelly may play an antibiotic role until embryogenesis is completed (Wake and Dickie, 1998).

Amphibian egg-jelly is composed of fibrous structures that form several sublayers (Good and Daniel 1943; Katagin 1965; Humphries 1966; Freeman 1968; Shivars and James 1970; Gusseck and Hedrick 1971; Yurewicz *et al*., 1975; MacLaughlin and Humphries 1978; Carroll *et al*., 1992; Omata 1993; Wake and Dickie 1998, Onitake *et al*., 2000, Fig. 1). Egg-jelly matrices are secreted in the posterior portion of the oviduct, pars convoluta. Each sublayer is accumulated by turns in the corresponding region of the pars convoluta when an ovulated egg passes through it (Jego *et al*., 1983a, b; Wake and Dickie, 1998; Okimura *et al*., 2001). It is known that the carbohydrate moieties are distinct among sublayers (Yurewicz *et al*., 1975; Bonnell and Chandler, 1996; Mozingo and Hedrick, 1999; Okimura *et al*., 2001), suggesting that each layer plays a distinct role in fertilization and development.

In the newt, *Cynops pyrrhogaster*, the egg-jelly is morphologically divided into six layers, which include the J0 to J4 layers of the inner region and the outermost sticky (st) layer (Onitake *et al*., 2000). Moreover, the J4 and st layers are composed of two distinct sublayers of carbohydrate components (Okimura *et al*., 2001).

The sperm-egg interaction occurs in the egg-jelly in the fertilization process of urodeles as with many animals. The urodele sperm are quiescently reserved in the spermatheca

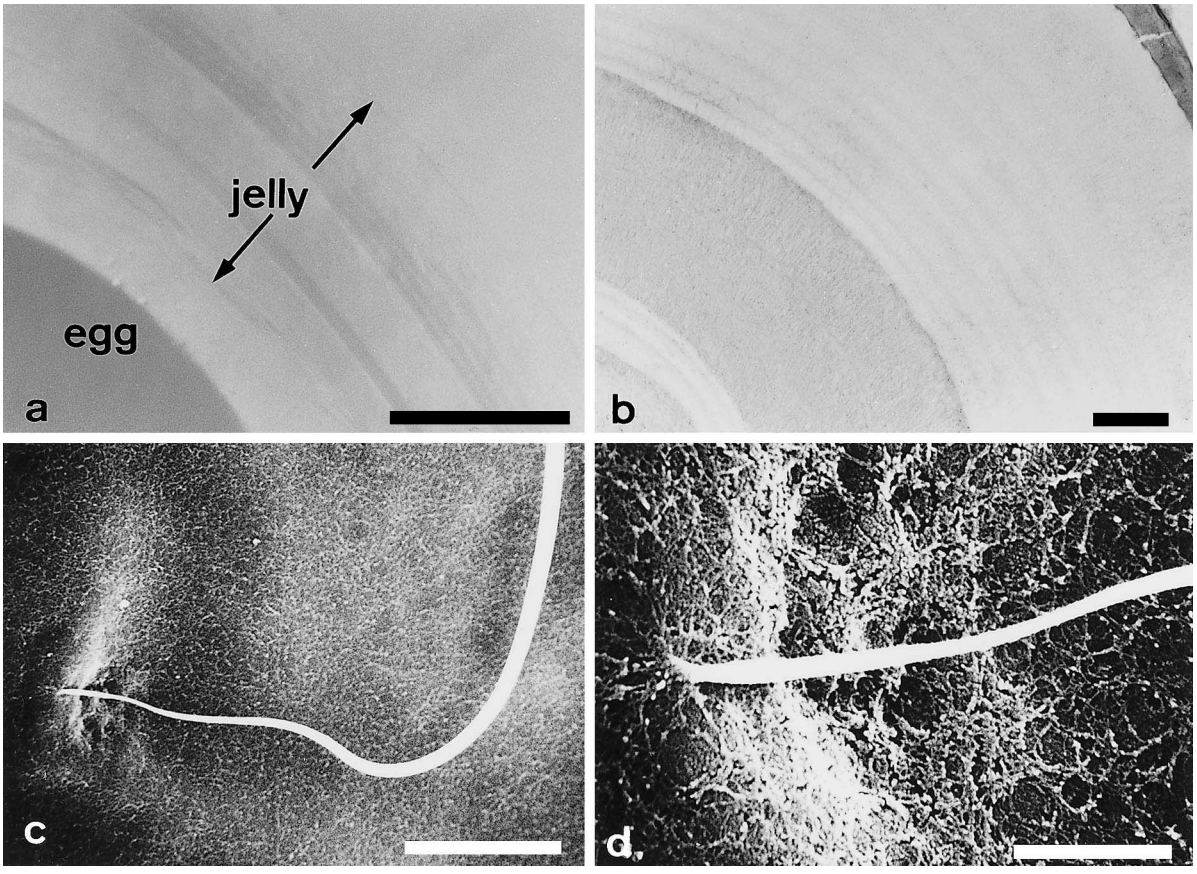


Fig. 1. Egg-jelly of *Cynops pyrrhogaster*. (a) A microscopic view of an egg-jelly. Sublayers of the egg-jelly were visible by the hydration. (b) A section of the egg-jelly. Egg-jelly of *C. pyrrhogaster* has six sublayers. Each sublayer has morphologically distinct features. (c and d). SEM observation of the surface of the egg-jelly of *C. pyrrhogaster*. Dry sperm were inseminated onto the surface of the jelly layer of the mature egg. The egg was fixed, dehydrated, spattered with platinum, and observed with SEM. (c) Sperm entering the egg-jelly. (d) High-magnification view of (c). The tip of the sperm head is penetrating the fibrous jelly matrix. Bar. 300 μm (a), 50 μm (b), 5 μm (c) and 1 μm (d).

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of females (Greven, 1998). They are directly inseminated on the surface of the egg-jelly and then begin to move. The activity for the initiation of sperm motility is localized in the st layer of the egg-jelly of *C. pyrrhogaster* (Watanabe *et al*., submitted), and a sperm-motility inducing substance (SMIS) is responsible for this activity (Ukita *et al*., 1999). The SMIS is a heat-stable protein approximately 50 kDa in molecular weight (Mizuno *et al*., 1999). An inactive form of more than 500 kDa also exists in the egg-jelly, suggesting that the SMIS is activated by sperm touching the surface of the egg-jelly during fertilization.

The cations in the egg-jelly are also involved in the activity initiating sperm motility. The egg-jelly of *C. pyrrhogaster* contains six detectable cations (Ukita *et al*., 1999). The potassium cation can induce the motility of *Cynops* sperm if they are treated at a high concentrations, although the estimated K^+ concentrations in the egg-jelly are not sufficient for sperm to induce motility (Ukita *et al*, 1999). It has also been suggested that K^+-channels are involved in the initiation of sperm motility in the egg-jelly (Watanabe *et al*., submitted). Thus, the initiation of sperm motility by the SMIS seems to be mediated by the influx of K^+ into the sperm in the egg-jelly. The calcium ion is also involved in the initiation of sperm motility by the SMIS. The egg-jelly of *C. pyrrhogaster* sustains approximately 6 mM Ca^{2+}. Calcium ions raise the activity for the initiation of sperm motility, though they cannot alone induce sperm motility (Ukita *et al*., 1999). When sperm treated with a Ca^{2+} blocker are directly inseminated onto the egg-jelly, they do not show any motility (Watanabe *et al*., submitted), indicating that the influx of Ca^{2+} into sperm is an inevitable event in the SMIS's initiation of motility. Therefore, those cations in the egg-jelly are a significant factor in the initiation of sperm motility.

In moving sperm, activation of motility appears to be induced by the cations. A recent study of Ca^{2+}channel in the KO mouse for the *Catsper* gene indicated that the influx of Ca^{2+} through the channel is involved not in the initiation but in the activation of sperm motility (Ren *et al*., 2001). Calcium ions in the egg-jelly may mediate the activation of sperm motility in *C. pyrrhogaster* as in the mouse, as it has been found to activate motility in *Cynops* sperm under experimental conditions (Watanabe and Onitake, 2003). The sodium ion, which is another monovalent cation detected in the egg-jelly (Ukita *et al*., 1999), can also induce sperm motility at higher concentrations than those estimated in the egg-jelly, and the motility of the moving sperm is activated independently of Ca^{2+} suggesting that Na^+ in the egg-jelly may also be involved in the activation of sperm motility in *C. pyrrhogaster*.

In urodeles, the sperm acrosome reaction has been found to be induced in the egg-jelly (Picheral, 1977; Sasaki *et al*., 2002). The activity for induction of the acrosome reaction is localized in the outer region of the egg-jelly of *C. pyrrhogaster* (Sasaki *et al*., 2002). The activity is concentrated in two peaks in the gel-filtration of the egg-jelly extract, and one of the peaks has been detected in the fraction of more than 500 kDa in SDS-PAGE (Onitake *et al*., 2000; Sasaki *et al*., 2002). Macromolecules forming the egg-jelly structure are the major substance in the fraction, suggesting that they contribute to induction of the acrosome reaction in the egg-jelly. The acrosome reaction-inducing substance is a glycoprotein, and both the protein portion and the carbohydrate moiety are responsible for its activity (Sasaki *et al*., 2002). The sperm acrosome reaction depends on the external Ca^{2+}, as with many animals (Sasaki *et al*., submitted). The estimated Ca^{2+} concentrations in the egg-jelly are sufficient for most sperm to induce the acrosome reaction (Ukita *et al*., 1999).

The egg-jelly structure of *C. pyrrhogaster* is responsible for guiding sperm to the surface of the vitelline envelope (Itoh *et al*., 2002). Sperm in solution move in a circle, whereas they move forward in the egg-jelly. Sperm with forward motility is observed in gelatin gel, which has been used as a substitute for the egg-jelly structure. When de-jellied eggs are inseminated with sperm through the gelatin gel, the fertilization efficiency is dramatically increased, suggesting that the gel structure is one of the major factors in the achievement of fertilization in the newt. In contrast, many sperm are trapped when passing through the egg-jelly (Itoh *et al*., 2002). Thus, the egg-jelly structure plays two opposing roles that either support sperm movement or exclude them from contributing to the fertilization.

Vitelline envelope

Vitelline envelope is the second egg-coat facing sperm in amphibian fertilization. In anurans, the envelope is formed in the ovary and partially digested by the protease in the anterior portion of the oviduct, pars recta, then acquiring the ability to bind to the sperm (Katagiri *et al*., 1999; reviewed by Katagiri, 1987 and Iwao, 2000). In fertilization, the vitelline envelope is modified by the contents released from the cortical granules and act as a block to polyspermy (Hedrick and Nishihara, 1991). In urodeles, the eggs are physiologically polyspermic, and some sperm enter the eggs during fertilization (Elinson, 1986). These findings suggest that the vitelline envelope has distinct feature between anurans and urodeles.

The vitelline envelope of *C. pyrrhogaster* acquires its sperm-binding ability in the oviduct (Nakai *et al*., 1999), suggesting that conversion of the vitelline envelope occurs in urodeles as in anurans. The vitelline envelope of the uterine egg binds only to the acrosome-reacted sperm, and the sperm-binding ability is missing in the vitelline envelope of the fertilized egg. Thus, the envelope may prevent excess sperm from participating in fertilization.

The vitelline envelope of *C. pyrrhogaster* is composed of six major components (Nakai *et al*., 1999) and three layers that can be detected immunologically (Fig. 2). The sperm binding to the vitelline envelope is mediated by heparin/heparan sulfate located on the surface of the acrosome-reacted sperm (Nakai *et al*., 1999). A substance of 84 kDa has been purified from the vitelline envelope with the hep-

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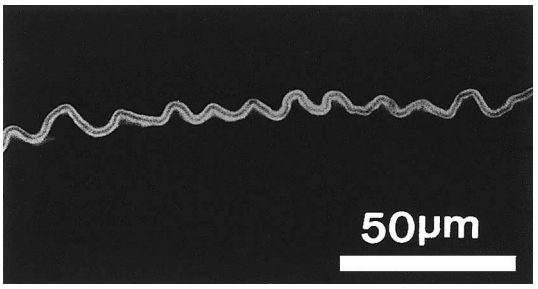


Fig. 2. Immunofluorescence staining of the vitelline envelope of *Cynops pyrrhogaster*. The vitelline envelope of *C. pyrrhogaster* was homogenized and injected into the gluteal muscle of a rabbit. The polyclonal antibody that binds to all of the major six substances in the vitelline envelope was obtained from blood. A section of the mature egg was immunoreacted with the antibody and then treated with the FITC-conjugated anti-rabbit IgG. The photograph shows a high-magnification view of the egg surface. The outer and inner surfaces of the vitelline envelope were stained with the antibody.

arin-affinity column, while a molecule of the same size has been detected in the outer portion of the vitelline envelope of the mature eggs with the anti-vitronectin antibodies (Suzuki and Onitake, unpublished data). It is known that a heparin-binding domain exists in vitronectin (Suzuki *et al*., 1984), indicating that it may be involved in the sperm-binding in *C. pyrrhogaster*.

ZPC molecules of 84 kDa and 70 kDa have also been detected in the inner portion of the vitelline envelope of *C. pyrrhogaster* (Makabe *et al*., 2003). The ZPC is the homologue of mouse ZP3 that is responsible for the first binding to sperm and induction of the acrosome reaction in the zona pellucida (Bleil and Wassarman, 1980), and it has been widely detected in the vitelline envelope of many vertebrates from fish to mammals (Ringuette *et al*., 1988; Kipersztok *et al*., 1995; Harris *et al*., 1994; Yang and Hedrick, 1997; Kubo *et al*., 1997; Murata *et al*., 1995; Chang *et al*., 1996). The ZPC is thought to contribute to the sperm binding in *Xenopus laevis* (Vo and Hedrick, 2000), as in many species. It cannot, however, contribute to the first binding of sperm to the vitelline envelope in *C. pyrrhogaster* because it is localized in the inner portion of the envelope. Regardless, the localization of the distinct molecules in the inner and outer portions suggests that the vitelline envelope of *C. pyrrhogaster* is a functionally organized matrix that may play a role in fertilization.

System equipped for internal fertilization in the newt egg-coat

Newts undergo fertilization in the cloaca of females. The ovulated eggs pass through the oviduct and are fertilized with sperm stored in the spermatheca. This fertilizing condition is distinct from that of external fertilization in at least two points. First are the stable surroundings of the egg during fertilization. In external fertilization, while the decrease in osmolality around the ejaculated sperm induces the initiation of motility (Inoda and Morisawa, 1987; Morisawa, 1994), the egg-jelly gradually swells in response to the hydration in water and finally prevents the sperm from passing through it (MacLaughlin and Humphnes, 1978). During the hydration, the soluble substances in the egg-jelly are gradually diffused into water, and the equipment required for the successful fertilization is being disrupted in the egg-jelly (Olson and Chandler, 1999). In contrast in the internal fertilization, no change occurs in the volume and equipment of the egg-jelly during fertilization. Thus, it appears that the inseminated sperm have enough time to pass through the egg-jelly while responding to every stimulus for the spenn-egg interaction.

In newt fertilization, SMIS in the egg-jelly triggers the initiation of sperm motility instead of an environmental change in osmolality (Fig. 3; Ukita *et al*., 1999; Watanabe *et al*., submitted). The function of SMIS in the initiation of sperm motility may be an adaptation of newt fertilization in response to the conditions of internal fertilization. This hypothesis is supported by the localization of SMIS activity in the outermost sublayer of the egg-jelly where sperm should begin to move at the start of fertilization (Watanabe *et al*., submitted). The outermost sublayer is added to the posteriormost portion of the oviduct, uterus (Okimura *et al*., 2001). It has been reported that the oviductal secretions are modified to fit the fertilization mode of a urodele (Greven, 1998). Similarly, the SMIS may be secreted in the uterus to fit with the internal mode of fertilization in urodeles. Moreover, newt sperm can respond to the low osmotic environment to initiate their motility as other amphibians do in external fertilization (Ukita *et al*., 1999). Our recent data indicate that the initiation of sperm motility caused by SMIS or low osmolality is controlled by distinct intracellular signaling cascades (Watanabe *et al*., submitted).

The other point that is specific to the internal fertilization of newts is the number of sperm inseminated onto the egg at fertilization. In external fertilization, ejaculated sperm are directly inseminated onto the surface of the spawned egg. Therefore, a large number of fresh sperm have the chance to contribute to the fertilization of each egg. In contrast, in internal fertilization, the number of sperm inseminated onto the surface of the egg-jelly is thought to be limited because sperm stored in the spermatheca are inseminated at fertilization. Furthermore, sperm are stored for a relatively long period before fertilization, and the ability to contribute to the fertilization is thought to be decreased in some sperm. The fertilization mechanism of the newt should be adapted to insemination of a limited number of sperm. Though this has also been hypothesized with regard to internal fertilization of urodele species (Elinson, 1976), the nature of the mechanism is wholly unknown.

In *C. pyrrhogaster*, when an aliquot of sperm is directly inseminated onto the surface of the jelly layer of the mature egg, the egg is easily fertilized (Matsuda and Onitake, 1984a, b). This is a good model system for investigating the system of internal fertilization. When eggs obtained from the oviduct are inseminated with dry sperm, the eggs are fertilized normally, even if not all sublayers of the egg-jelly

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surround the egg surface (Takahashi *et al*., submitted). However, most eggs develop abnormally in every condition. Whereas, when the reduced number of sperm is inseminated onto those eggs, only the eggs with full sublayers are fertilized and develop normally with high efficiency (Takahashi, submitted). In the latter experiment, approximately 600 sperm are inseminated onto each egg, which is very close to the number in the jelly layers of normally spawned eggs (Takahashi *et al*., submitted). This result indicates that the fertilization system with the insemination of a limited number of sperm is made possible by the egg-jelly.

These observations indicate that the outermost layer of the egg-jelly sublayer is crucial to fertilization in the newt. As mentioned above, the activity required for induction of the acrosome reaction and the activity for the initiation of sperm motility is localized in the outermost sublayer (Sasaki *et al*., 2002; Watanabe *et al*., submitted). The fertilization is not inhibited when the eggs are inseminated with acrosome-intact sperm which are induced motility (Takahashi *et al*., submitted). Thus, the timing to induce the acrosome reaction may be important for achieving successful fertilization in *C. pyrrhogaster.*

The sperm acrosome reaction is induced near or at the vitelline envelope in *Bufo* and *Xenopus* (Fig. 3; Yoshizaki and Katagiri, 1982; Barisone *et al*., 2002; Ueda *et al*., 2002). In *Bufo arenarum*, the acrosomal vesicle is maintained in the egg-jelly substance (Arranz and Cabada, 2000), and the sperm acrosome reaction is induced at the vitelline envelope. In *Xenopus laevis*, the activity required for induction of the sperm acrosome reaction is localized in the extract of the anteriormost portion of the oviduct, pars recta (Ueda *et al*., 2002). These results suggest that the substance inducing the acrosome reaction is added to the vitelline envelope prior to being surrounded by the egg-jelly. In contrast, in a primitive anuran, *Discoglosus pictus* and a urodele, *Pleurodeles walti*, as well as *C. pyrrhogaster*, the sperm acrosome reaction is observed in the egg-jelly (Picheral, 1977; Campanella *et al*., 1997). Thus, the site to induce the sperm acrosome reaction is distinct among amphibian species. A similar variation regarding the induction sites for the acrosome reaction is seen in mammalian species. In the mouse, the acrosome reaction is induced in the zona pellucida, which corresponds to the vitelline envelope of the amphibian egg (Bleil and Wassarman, 1980). In contrast, in some other species, including the hamster and rabbit, it is at least partially induced in the cumulus (Cummunis and Yanagimachi, 1982; Bedford, 1998), though fertilization occurs without it. In the musk shrew, *Suncus murinus*, the cumulus is the obvious inducer of the sperm acrosome reaction (Bedford *et al*., 1997) and is crucial to the successful fertilization. The acrosome-reacted sperm selectively kill the surrounding cumulus cells in penetrating the ball of cumulus cells and finally attach to the zona pellucida. Thus, the timing and role of the sperm acrosome reaction may be distinct among mammalian species (Bedford, 1998; Kaneko *et al*., 2001). The timing to induce the sperm acrosome reaction is also thought to be responsible for the species-specific function in the amphibian fertilization. As mentioned above, the acrosome-reacted sperm passing through the egg-jelly bind to the vitelline envelope in *C. pyrrhogaster* (Fig. 3), whereas

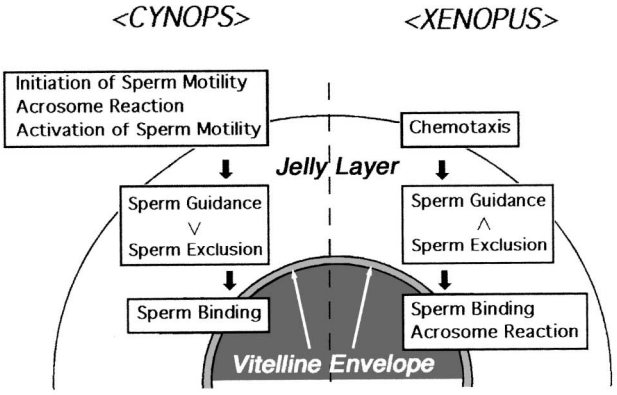


Fig. 3. The sites to induce the sperm-egg interactions in egg-coats of the newt, *Cynops pyrrhogaster* and the frog, *Xenopus laevis*. The upper boxes show the events induced on the surface of the egg-jelly. The middle boxes show those in the egg-jelly. Although sperm guidance and sperm exclusion are provided by the structure of egg-jelly in both species, the structure dramatically raises the fertilization efficiency in *C. pyrrhogaster*. Because fertilization occurs externally in *Xenopus*, sperm exclusion occurs by hydration of the egg-jelly with the passage of time. The bottom boxes show the events induced at the vitelline envelope.

in *Xenopus* and *Bufo*, sperm induce the acrosome reaction and bind to the vitelline envelope at the same site. Thus, in anurans, responsiveness to the signal inducing the acrosome reaction may be a crucial mediator of fertilization for each sperm. It has actually been reported that, in *Bufo*, it takes 10 min for the acrosome reaction to be induced in most competent sperm (Barisone *et al*., 2002). In contrast, responsiveness to the signal does not affect the chance of each sperm to contribute to the fertilization in *C. pyrrhogaster* because the acrosome reaction has already been induced in most sperm passing through the egg-jelly. The distinct site at which the sperm acrosome reaction is induced may characterize the fertilization strategy of each species.

In the internal fertilization of urodeles, fertilization must be achieved with a limited number of sperm. Although the fertilization mode may cause fertilization failure because of the lack of sperm, urodeles appear to have a fertilization system adapting to the severe condition. The localization of the activities for the sperm-egg interactions may be one of the major factors responsible for the adapted system of *C. pyrrhogaster*. Most *C. pyrrhogaster* sperm have already acquired in vas deferens the ability to respond to the native signals for the sperm-egg interaction such as induction of the acrosome reaction and the initiation of motility (Ukita *et al*., 1999; Onitake *et al*., 2000; Sasaki *et al*., 2002; Watanabe and Onitake, 2003). Then, most sperm attached to the outermost sublayer of egg-jelly have adequate time to begin to move and complete the acrosome reaction

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before reaching the vitelline envelope. The egg-jelly of *C. pyrrhogaster* may enable most inseminated sperm to contribute to fertilization rather than to eliminate excess (probably inferior) sperm.

The structure of the egg-jelly also acts to raise the chance of each sperm to contribute to fertilization in *C. pyrrhogaster* because it strongly affects the fertilization efficiency of mature eggs (Itoh *et al*., 2002; Fig. 3). In anurans, *Xenopus* and *Bufo*, fertilization is achieved in a solution (Katagiri, 1966; Olson and Chandler, 1999), suggesting that the dependency on the structure of the egg-jelly is greater in *Cynops* than the anurans. Indeed, *Cynops* sperm have distinct features from those of the anurans with regard to size and the manner of movement. The function of the egg-jelly structure may be crucial to successful fertilization in response to insemination with a limited number of sperm.

The vitelline envelope of *C. pyrrhogaster* is composed of three qualitatively distinct layers (Fig. 2). A layered composition of the vitelline envelope has been reported in some species. In the mouse, the zona pellucida contains different oligosaccharide chains in the inner and outer regions, which are supposed to play a role with regard to the sperm penetrating it (Avilés *et al*., 1999). The layered structure of the vitelline envelope has been ultrastructurally demonstrated in the egg of *Xenopus laevis* (Larabell and Chandler, 1988a, b). The envelope is composed of two layers, which are separated to form the fertilization envelope at fertilization. Although the role of each layer of the vitelline envelope is unknown in *C. pyrrhogaster*, each layer may play a distinct role that reflects other features of newt fertilization.

Egg-coats of *C. pyrrhogaster* have a layered composition, with each layer containing specific molecules. The sperm-egg interactions are induced in the restricted layers, and most inseminated sperm appear to be potent in contributing to the fertilization (Fig. 3). The egg coat is thought to guide sperm to the egg surface while maintaining the fertilization ability, which results in the sperm having a chance to contribute to a successful fertilization. This system may allow species to reproduce offspring with certainty through internal fertilization, with sperm facing less competition than those of amphibian species undergoing external fertilization because the number of spenn inseminated onto an egg at fertilization is limited. The comparative data described above between anurans and urodeles suggest that the combination of sites for sperm-egg interactions, may characterize the fertilization strategy in amphibian species. This kind of comparative study of fertilization systems among species should lead to a new understanding of how the varieties of amphibian fertilization have been established.

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