Chapter 8
Acrosome Reaction-Mediated Motility Initiation That Is Critical for the Internal Fertilization of Urodele Amphibians

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Abstract The reproductive modes of extant amphibians are highly diversified in their adaptation to species-specific reproductive environments. In the external fertilization of most amphibians, fertilization begins from motility initiation of the sperm by the hyposmolality of freshwater at spawning, whereas in internal fertilization, the sperm initiate motility without any change in osmolality. Acrosome reaction (AR)-mediated motility initiation is an initial event of the internal fertilization of the urodele amphibian *Cynops pyrrhogaster*, also known as the Japanese fire belly newt. It initiates motility of female-stored sperm on the surface of the jelly layer in the cloaca. This unique mechanism of motility initiation is based on the fine structure of the jelly matrix, in which AR-inducing substance (ARIS) and sperm motility-initiating substance (SMIS) are localized in the sheet-like structure covering the outer surface and in the granules beneath it. The ARIS and the SMIS repeatedly increase the sperm intracellular Ca^{2+} level and result in the sporadic initiation of motility within 4 min. The SMIS activity is also present in the jelly layer of the externally fertilizing anuran amphibian *Discoglossus pictus* (the Mediterranean painted frog), but a thick layer of matrix simply covers the outer surface of the jelly layer. The AR-mediated mechanism may be established in the reproductive strategies for the internal fertilization of urodeles.

Keywords Amphibians • Acrosome reaction • Extracellular matrix • Fertilization • Sperm motility

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8.1 Diversity of Reproductive Modes in Amphibians

Amphibian reproduction adapts to various environments in freshwater and on land. The variety of reproductive modes among amphibians has been expected to provide models for the study of unknown mechanisms underlying reproduction (Wake and Dickie 1998). Duellman and Trueb (1994) proposed the diversification path of reproductive modes in amphibian evolution. Fertilization occurs in lentic freshwater in the primitive mode of amphibian reproduction, and eggs and sperm are simultaneously spawned in freshwater. The sperm initiate motility in response to the hyposmolality in the freshwater and fertilize the eggs.

Amphibian eggs are surrounded by an oviduct-secreted matrix called the jelly layer. A variety of modifications are seen in the morphology and physiology of the oviductal secretion, which assure reproduction under a species-specific environment. For example, the fertilization of some anurans occurs in lotic freshwater. For the adaptation to water flow, the jelly layer is modified to form network-forming morphology, as seen in the frog *Rana tagoi* (Fig. 8.1a). In a more evolved mode of anuran reproduction, fertilization occurs arboreally. In this case, a foam nest is often formed with an oviductal secretion, providing the fertilization environment on the tree, as seen in the treefrog *Rhacophorus arboreus* (Fig. 8.1b).

Internal fertilization is the most evolved mode of amphibian reproduction. It is seen in a few anuran species, more than 90 % of urodele species, and all caecilian species. In urodeles, the internal fertilization evolved from external fertilization (Duellman and Trueb 1994). In the internal fertilization of urodeles, sperm are quiescently stored in the spermatheca, the female sperm reservoir in the cloaca, for months (Duellman and Trueb 1994; Greven 1998). At the beginning of fertilization, the sperm are inseminated on the surface of the jelly layer. The jelly layer is responsible for sperm motility initiation without a hyposmotic condition, as seen in the newt *Cynops pyrrhogaster* (Ukita et al. 1999).

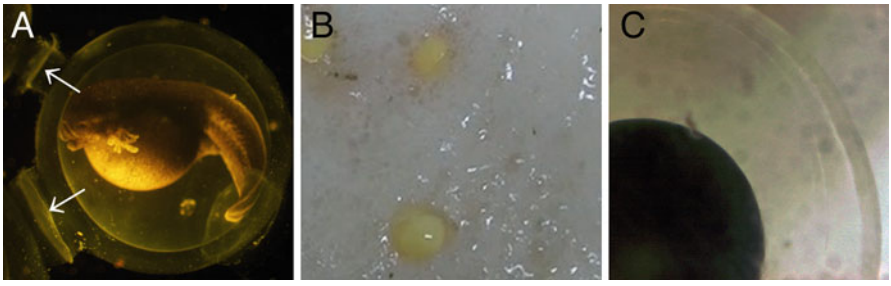

Fig. 8.1 Modification of oviductal secretion in amphibians. **(a)** Jelly layer of *Rana tagoi*. *Arrows* indicate joint regions between the eggs. **(b)** Foam nest of *Rhacophorus arboreus*. **(c)** Jelly layer of *Cynops pyrhogaster*

8.2 The Jelly Layer of Amphibian Eggs

The jelly layer of amphibian eggs is a gelatinous, sugar-rich matrix surrounding the eggs as the outermost egg coat. It is a multifunctional structure for attachment of fertilized eggs to the substrates, the physical barrier for developing embryos, defense against bacterial invasion, and fertilization (Wake and Dickie 1998). The jelly layer is composed of several sublayers, each of which contains qualitatively and quantitatively different components (Fig. 8.1c; Greven 1998; Okimura et al. 2001). The components are secreted in a specific compartment of the pars convoluta, a distal portion of the oviduct, and sometimes in the most proximal portion of the oviduct (called the uterus or ovisac). They are accumulated on the surface of the vitelline envelope to form each sublayer (from the inner to outer) in ovulated eggs passing through the oviduct.

The role of the jelly layer in amphibian fertilization has been investigated for many years. It is a source of divalent cations for the sperm–egg interaction (Katagiri 1987; Takayama-Watanabe et al., in press), and proteinaceous components also have significant roles in the interaction (Arranz and Cabada 2000; Olson et al. 2001; Watanabe et al. 2009, 2010). Interestingly, some components such as AR-inducing substance (ARIS) seem to localize in a species-specific manner (Watanabe and Onitake 2002), suggesting that the function of the jelly layer in fertilization is modified among amphibians.

In urodeles, fertilization is strongly dependent on the outer sublayer(s) of the jelly layer (MacLaughlin and Humphries 1978; Takahashi et al. 2006). Activities for sperm AR induction and motility initiation localize in the outermost sublayer of the newt *C. pyrrhogaster* (Sasaki et al. 2002; Watanabe et al. 2003). Takahashi et al. (2006) reported that sperm AR on the surface of the jelly layer is critical for the fertilization of *C. pyrrhogaster* because the insemination of AR-induced sperm, in contrast to that of AR-intact sperm, remarkably reduced fertilization rates. In the anurans *Bufo japonicus* and *Xenopus laevis*, because the AR is induced after sperm passing through the jelly layer (Yoshizaki and Katagiri 1982; Ueda et al. 2002), a specific mechanism for the sperm–egg interaction may be present on the surface of the jelly layer of *C. pyrrhogaster,* and it may be responsible for the success of internal fertilization.

8.3 Acrosome Reaction-Mediated Motility Initiation

Acrosome reaction-mediated motility initiation is the mechanism underlying the induction of motility of female-stored sperm in the internal fertilization of *C. pyrrhogaster* (Watanabe et al. 2010). This initiation is based on the fine structures of the jelly surface, where a sheet-like structure covers the outer surface of the egg jelly and sequesters many granules from outside (Watanabe et al. 2010; Fig. 8.2a). By immunological localization, sperm motility-initiating substance (SMIS) and ARIS

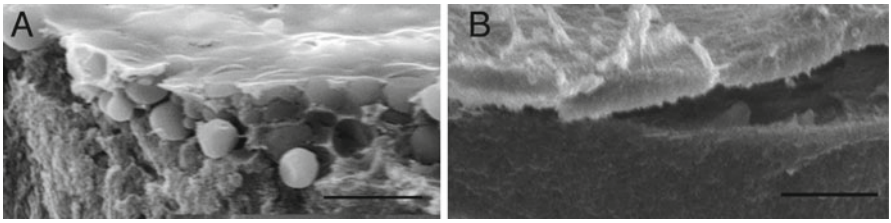

Fig. 8.2 Fine structures on outer surface of amphibian jelly layer. **(a)** *Cynops pyrrhogaster*. **(b)** *Discoglossus pictus*

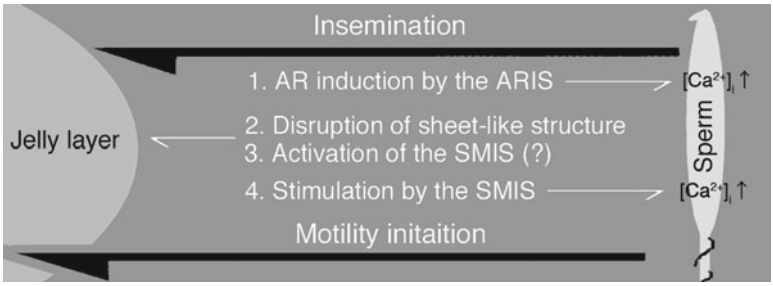

Fig. 8.3 Sperm–egg interaction on the surface of the jelly layer in *Cynops pyrrhogaster*. *ARIS* acrosome reaction-inducing substance, *SMIS* sperm motility-initiating substance

exist in the sheet-like structure and the granules, respectively. At the beginning of internal fertilization, the large egg size of urodeles results in outspread the external opening of the spermatheca in the cloaca, and the quiescently stored sperm are mechanically inseminated onto the surface of the egg jelly (Fig. 8.3). The sperm immediately (<30 s) undergo the AR in response to the ARIS in the sheet-like structure (Watanabe et al. 2011). The enzymes released from the acrosomal vesicle are suggested to disrupt the sheet-like structure and expose the SMIS in the granules to the jelly surface (Watanabe et al. 2010). The SMIS in the jelly layer is largely inactivated by the association with jelly substances (Mizuno et al. 1999), and recent data from our laboratory indicate that a sperm protease may mediate the activation of the SMIS (Yokoe et al., unpublished data). The active SMIS, in turn, stimulates sperm to initiate motility. In contrast to the AR, motility initiation occurs sporadically among sperm within 1–4 min after insemination (Watanabe et al. 2003, 2011).

The isolation of the SMIS in the jelly layer is unique to AR-mediated motility initiation. Although sperm motility is controlled by a variety of extracellular cues such as osmotic conditions, steroids, peptides, and proteases (Morisawa et al. 1999; Miyata et al. 2012) in animal species, all are ready to affect sperm by themselves at fertilization. The exposure of the SMIS and its activation fully depends on the inseminated sperm, which should be one of the reasons why sporadic motility initiation occurs through the AR-mediated mechanism. Sporadic motility initiation is also caused by valid levels of intracellular Ca^{2+} of the quiescently stored sperm (Watanabe et al. 2011).

In the sperm of *C. pyrrhogaster*, intracellular Ca^{2+} is increased in both AR induction and motility initiation, as in the sperm of other animal species (Darszon et al. 2006), and immediately drops down to the initial level (Watanabe et al. 2011). In addition, we recently found that subsequent influxes of Ca^{2+} in the motility-initiated sperm cause a high motility state (Takahashi et al. 2013). Activation of the motility state is needed for sperm to propel through the viscous jelly matrix. Conversely, AR-mediated motility initiation and the subsequent activation of the motility state are potent to exclude sperm, diminishing their ability to appropriately control the intracellular Ca^{2+} level. Actually, many sperm are left on the surface of the jelly layer in naturally spawned eggs of *C. pyrrhogaster* (Takahashi et al. 2006). It is interesting that the insemination of too many sperm can achieve fertilization without AR-mediated motility initiation (Takahashi et al. 2006). Because AR induction is essential for sperm to interact with the vitelline envelope (Nakai et al. 1999), fertilization is thought to occur by abnormal sperm that spontaneously miss the acrosome and initiate motility (Watanabe and Onitake 2003). In that case, most of the fertilized eggs fail in normal development because of polyspermy, although the eggs of *C. pyrrhogaster* are physiologically polyspermic (Street 1940). Such abnormalities of sperm physiology are commonly seen in sperm from every male (Watanabe and Onitake 2003) and are thought to be increased during the months of female sperm storage. AR-mediated motility initiation may have a role in controlling the amount of sperm participating in fertilization to ensure the success of embryonic development.

8.4 SMIS Activity in the Amphibian Jelly Layer

Sperm motility-initiating substance (SMIS) activity in egg jelly is crucial to initiate motility in the internal fertilization of urodele amphibians. How SMIS-triggering motility initiation is originated in the amphibian fertilization system may be significant to understand the diversification mechanism for the amphibian mode of reproduction. To address this question, we performed comparative studies using the anti-SMIS monoclonal antibody (Ohta et al. 2011; Takayama-Watanabe et al. 2012). SMIS activity is present in the jelly layers of the externally fertilizing urodele *Hynobius lichenatus* and the anuran *Discoglossus pictus*, indicating that SMIS activity is widely conserved among urodeles and anurans. In the primitive anuran *D. pictus*, the acrosome reaction (AR) is induced in the outermost jelly sublayer (Campanella et al. 1997), although a thick layer of jelly matrix covers the outer surface without locating granules (Takayama-Watanabe et al. 2012; Fig. 8.2b). In addition, no sporadic feature is seen in sperm motility initiation by the jelly substances, suggesting that the SMIS activity has a distinct role in the fertilization of *D. pictus*. Because *D. pictus* sperm initiate motility in response to hyposmolality, the SMIS activity seems to be redundant for the motility initiation at their external fertilization. It is well known that mammalian sperm show a specific motility state to propel through the oviductal matrix (Yanagimachi 1994). It is suspected that the SMIS activity in the jelly layer of *D. pictus* is for the activation of the sperm motility state in relationship to the penetration of the jelly matrix.

8.5 Perspective

The SMIS is a key for the AR-mediated motility initiation critical for the success of internal fertilization of urodeles. It acts based on the fine structure of the egg jelly surface at the beginning of fertilization. Modifications of the jelly matrix-based mechanism may be an engine for the adaptation of motility control of amphibian sperm to various environments. Diversification of reproductive mode widely occurs among animal species and contributes to the success of fertilization under a specific condition. Every mode of reproduction sometimes looks so specific that we know little about the molecular mechanism for its diversification. The SMIS-triggering mechanism is potent to reveal the plasticity of sperm motility control that correlates with the diversification of reproductive modes in amphibian species.

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