Maternal control of gamete choice during fertilization

SREELAJA NAIR*

Department of Biological Sciences, Tata Institute of Fundamental Research, Colaba, Mumbai, India

ABSTRACT **Sexually reproducing organisms generate male and female haploid gametes, which meet and fuse at fertilization to produce a diploid zygote. The evolutionary process of speciation is achieved and maintained by ensuring that gametes undergo productive fusion only within a species. In animals, hybrids from cross-species fertilization events may develop normally, but are usually sterile (Fitzpatrick, 2004). Metazoan sperm and eggs have several features to ensure that the gametes, which have evolved independently and also in conflict with each other, are competent to undergo fertilization (Firman, 2018). Fertilization is a specific process that is ideally supposed to result in randomized fusion of compatible egg and sperm. Here, I will discuss key processes driven by maternal factors in the egg that dictate earliest stages of gamete recognition, gamete choice and fusion in metazoans.**

KEY WORDS: *egg, sperm, chemotaxis, rheotaxis, GPI-anchored proteins, meiosis*

This review is an attempt to summarize recent studies that have provided exciting fundamental insights into the process of fertilization in metazoans. Textbooks typically describe fertilization as "the fusion of male and female gametes to form a zygote". This simple sentence encompasses the molecular and physical mechanisms of egg chemoattractants, sperm chemotaxis, physical properties of sperm and egg membranes, receptor-ligand interactions that allow egg-sperm fusion and the evolution of these processes. In the following sections, I discuss the diversity of the initial events in animal egg and sperm recognition and fusion that ensure fertilization in sexually reproducing metazoans is successful and yet species specific.

Maternal factors from eggs that guide sperm chemotaxis

The earliest demonstration of maternal control over fertilization in animals emerged when F.R. Lillie showed that eggs release an agglutination and aggregation agent to which the sperm of that species responds (Lillie, 1912; Lillie, 1913). Lillie used seawater in which unfertilized *Arbacia punctulata* eggs had been incubated and added a few drops of it into a suspension of motile sperm (Lillie, 1912). When *A. punctulata* egg water was added to motile *A. punctulata* sperm, the sperm clumped in a couple of minutes, a phenomenon Lillie termed as iso-agglutination. In addition to echinoderms, Lillie demonstrated the same phenomenon in annelids using the marine worm, *Nereis* (Lillie, 1912). Nereis egg water was unable to clump *A. punctulata* sperm, demonstrating species specificity and *A. punctulata* egg water rendered the *Ne-*

reis sperm clumped and non-motile, demonstrating cross-species toxicity (Lillie, 1912). This was perhaps the first demonstration of egg defense mechanisms to ensure reproductive isolation in a species. As an aside, Lillie also inferred that the *A. punctulata* egg water contained an agent to which the *A. punctulata* sperm were attracted, because a drop of the egg water in the sperm solution resulted in the formation of a ring of clumped sperm at the edge of the egg water drop, followed by a zone of clearance. Thus, the basic principles of how an animal egg might ensure fertilization by the same species sperm were uncovered.

Ironically, the clumping phenomenon that Lillie chose to study was shown to be possible by the high density of sperm used and the hydrodynamic forces generated by the sperm tails during motility (Collins, 1976; Riedel *et al.*, 2005). The chemoattractant hypothesis, which Lille never pursued, endured the test of time and has been identified as a likely mechanism of species-specific gamete interaction in invertebrates and vertebrates. Approximately 70 years after Lillie first posited, Speract/Resact, a short peptide was identified from the external jelly layer of sea urchin eggs and was shown to be the chemoattractant to which sea urchin sperm responded (Hansbrough and Garbers, 1981; Ward *et al.*, 1985). Speract/Resact was shown to bind a Guanylate cyclase receptor on the sperm flagellar membrane and increase intracellular levels of cyclic GMP, which potentiated the movement of the sperm towards the eggs, eventually enabling the acrosome reaction in

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*Address correspondence to: Sreelaja Nair. Department of Biological Sciences, Tata Institute of Fundamental Research, Homi Bhabha Road, Colaba, Mumbai, 400005, India. E-mail: s.nair@tifr.res.in - ![ORCID] https://orcid.org/0000-0001-7309-339X - web: https://www.tifr.res.in/~s.nair/Home.html

sperm heads (Suzuki *et al.*, 1984; Singh *et al.*, 1988).

It is intuitive that gametes that encounter each other in open water must have evolved mechanisms to ensure conspecies fertilization by attracting the correct partner gametes and to be doubly sure, eliminate gametes from other species present in the vicinity that may interact with the eggs. However, the ability of maternal peptides to steer sperm to an increasing concentration gradient towards the egg is not restricted to aquatic species that undergo external fertilization but is also found in ascidians and mammals (Yoshida *et al.*, 2002; Spehr *et al.*, 2003; Fukuda *et al.*, 2004; Guidobaldi *et al.*, 2008). Indeed, the molecular repertoire of maternal factors in eggs that trigger sperm chemotaxis include peptides, glycoproteins, fatty acids, hormones and odors (Olson *et al.*, 2001; Riffell *et al.*, 2002; Yoshida *et al.*, 2002; Zatylny *et al.*, 2002; Spehr *et al.*, 2003; Fukuda *et al.*, 2004; Bohmer *et al.*, 2005; Guidobaldi *et al.*, 2008; Yanagimachi *et al.*, 2013; Yoshida *et al.*, 2018) (Table 1). Interestingly, gametes of the same species have not co-evolved co-operative mechanisms for gamete recognition and often have co-evolved antagonistic mechanisms, perhaps to deter polyspermy (Firman, 2018). The molecular diversity of maternally encoded egg chemoattractants and its evolutionary distribution across metazoans conjures an erroneous image that sperm chemotaxis can ensure species-specific gamete fusion as long as the right gametes are attracted. However, there are several examples wherein maternal factors from mammalian eggs can trigger heterospecies sperm chemotaxis (Sun *et al.*, 2003; Teves

et al., 2006; Guidobaldi *et al.*, 2008).

Since mammals undergo internal fertilization, the role of sperm chemotaxis, especially because it is not species specific, remains obscure. In mammals, of the ~10^7 sperm deposited in the female, only ~10% are chemotactically responsive (Barratt and Cooke, 1991; Williams *et al.*, 1993; Giojalas and Rovasio, 1998; Oliveira *et al.*, 1999). Sperm must undergo a series of biochemical and physiological changes known as capacitation, to become fertilization competent (Stival *et al.*, 2016). Separation of sperm subpopulations based on chemotaxis revealed that the chemotactically responsive population was enriched with capacitated sperm and the physiological state of capacitated, chemotactic sperm was transient (Cohen-Dayag *et al.*, 1994; Ralt *et al.*, 1994; Cohen-Dayag *et al.*, 1995). In mammals, sperm chemotaxis in response to maternal factors released by the egg is hypothesized to ensure that fertilization competent spermatozoa are available at the time of ovulation. This idea is partially supported by the observation that mammalian sperm chemotactic response can only be elicited from follicular fluids from follicles which contain mature, fertilizable eggs (Ralt *et al.*, 1991). The molecular mechanisms that allow maternal factors to guide sperm chemotaxis is best studied for Speract/Resact and its cognate Guanylate cyclase receptor (Darszon *et al.*, 2008). The phenomena of sperm capacitation, changes in intracellular Ca^{++} levels, sperm cell membrane depolarization etc that have been experimentally proven in sea urchins hold true across several species, including mammals. Despite successfully executing the

TABLE 1

**KNOWN MOLECULAR INTERACTIONS BETWEEN PROTEINS ON THE EGG
AND SPERM REQUIRED FOR FERTILIZATION IN DIVERSE METAZOAN SPECIES**

Process	Species	Chemoattractant Identity	Molecular Nature	Source	Chemoattractant receptor	Location
Sperm chemotaxis towards egg-derived factors	*Sepia officinalis*	SepSAP (Sepia sperm attracting peptide)	Peptide	Released from egg	Not Known	Not Known
	Haliotis rufescens	L-Tryptophan	Amino acid	Released from egg	Not Known	Not Known
	Asterias amurensis	asterosap	Peptide	Egg jelly layer	Not Known	Not Known
	Arbacia punctulata	Resact	Peptide	Egg jelly layer	Guanylate cyclase	Sperm flagella membrane
	Strongylocentrotus purpuratus	Speract	Peptide	Egg jelly layer	Guanylate cyclase	Sperm flagella membrane
	Ciona intestinalis	SAAF (sperm activating and attracting factor)	Sulfated Steroid	Released from egg	PMCA (plasma membrane Ca2+-ATPase)	Sperm flagella membrane
	Ciona savignyi	SAAF (sperm activating and attracting factor)	Sulfated Steroid	Released from egg	PMCA (plasma membrane Ca2+-ATPase)	Sperm flagella membrane
	Xenopus laevis	Allurin	Peptide	Egg jelly layer	Not Known	Not Known
	Xenopus tropicalis	Allurin	Peptide	Egg jelly layer	Not Known	Not Known
	Herrings, Flounders	SMIF (sperm motility initiation factor)	High molecular weight glycoprotein	Chorion membrane near micropyle on egg	Not Known	Not Known
	Mus musculus	Not Known	Not Known	Follicular and Oviduct Fluid, Cumulus cells, eggs	MOR23 (odorant receptor)	Sperm flagella membrane
	Rabbits	Progesterone	Hormone	Follicular and Oviduct Fluid, Cumulus cells, eggs	Not Known	Not Known
	Homo sapiens	Not Known	Not Known	Follicular and Oviduct Fluid, Cumulus cells, eggs	hOR17-4 (odorant receptor)	Sperm flagella membrane
	Homo sapiens	Not Known	Not Known	Follicular and Oviduct Fluid, Cumulus cells, eggs	hOR17-2 (odorant receptor)	Sperm flagella membrane
Sperm-Egg binding and fusion	*Danio rerio*	Bouncer	GPI-anchored Ly6/urokinase-type plasminogen activated receptor	Egg membrane	Not Known	Not Known
	Reptiles and Mammals	Not Known	Not Known	Not Known	SPACA4/SAMP14 (sperm acrosome membrane-associated protein 4/sperm acrosomal membrane protein 14)	Sperm head membrane
	Mus musculus, Homo sapiens	CD9	Tetraspanin protein family	Microvillar membrane on egg	Not Known	Not Known
	Mus musculus, Homo sapiens	Juno	GPI-anchored folate receptoe	Egg membrane	Izumo (Immunoglobulin protein family)	Sperm head membrane

essential biochemical changes to become fertilization competent and finding the correct egg, fertilization will fail if the sperm cannot bind to the egg and fuse. Thus in organisms such as frogs, fish and mammals the attention has shifted to identifying the cognate egg-sperm proteins that facilitate the actual binding and fusion of the sperm to the egg.

Physical principles of sperm chemotaxis

Sperm chemotaxis guided by maternal factors from the egg was discovered over 100 years ago. In species that undergo external fertilization, the phenomenon is easy to track visually and manipulate biochemically and has enabled molecular mechanisms of sperm-egg interaction to be elucidated. However, in such experimental conditions, the density of sperm around eggs is in vast excess than would be possible in open water. In species that undergo internal fertilization, though there is evidence of sperm chemotaxis, its actual physiological role remains unclear. In addition to chemical cues, physical principles also facilitate sperm movement to ensure sperm-egg interaction in both internally and externally fertilizing species. Both human and mice sperm demonstrate positive rheotaxis i.e. the ability to orient and swim against fluid flow. Recent *in vivo* studies in mice show that fluid secretion in the oviduct increases upon coitus and both mice and human sperm exhibit rotational movement and positive rheotaxis in the oviduct (Miki and Clapham, 2013). Both capacitated and noncapacitated mammalian sperm exhibit positive rheotaxis, which requires CatSper (Cation Channels of Sperm) activity (Miki and Clapham, 2013). This raises the possibility that positive rheotaxis would enable only the motile sperm to progress through the female reproductive tract, undergo capacitation and chemotactically respond to maternal egg factors. Positive rheotaxis microfluidic devices allow selective isolation of motile sperm (Zaferani *et al.*, 2018). Such microfluidic devices may eventually replace techniques such as density gradient centrifugation that are currently used to enrich quality sperm in Assisted Reproductive Technologies.

Interestingly, sea urchin sperm do not demonstrate positive rheotaxis and move similar to mouse sperm lacking CatSper channels (Miki and Clapham, 2013). So, how do sea urchin sperm reach the egg encountering varying levels of maternal egg chemoattractants on its way? Recent experiments with photoactivatable caged Resact allows sculpting of defined 3D chemoattractant profiles along which sea urchin sperm chemotaxis can be studied. Sea urchin sperm move along a helical axis in response to chemoattractants, which allow gradient sensing perpendicular to the axis of movement (Jikeli *et al.*, 2015). The helical navigation results in two distinct steering behaviors: smooth, incremental adjustments to the helical bend over time, which keeps the sperm on course and a second sharp, rapid change in the helical bend, which suddenly aligns an off-course sperm in the right general direction (Jikeli *et al.*, 2015). This second sharp change in the helical bend is interpreted as decision making and in simulation experiments confers an advantage to sperm to bend their helical paths in low signal to noise environments, such as low concentrations of maternal egg factors guiding sperm chemotaxis in open water (Kromer *et al.*, 2018). Thus, sea urchin sperm chemotaxis is emerging as an interesting paradigm to understand how biological systems ensure desired outcomes reliably, despite noisy guidance cues.

Female meiosis and biased fertilization outcomes

Employing chemotactic and physical principles discussed above, the general belief is that egg-sperm fusion at fertilization is insensitive to allelic composition in the gametes (Crow, 1991). Yet, from flies to humans, allelic combinations appear in proportions that defy the expected ratios if a particular egg-sperm fusion were a random event (Pimpinelli and Dimitri, 1989; Zollner *et al.*, 2004; Botta *et al.*, 2005; Larracuente and Presgraves, 2012; Didion *et al.*, 2015). Recent evidence indicates that female meiotic drive can bias the outcome of sperm choice and fertilization outcomes. The canonical view of female meiosis involves homologous chromosome separation during meiosis I, followed by sister chromatid separation during meiosis II. Genome-wide SNP mapping of meiotic products after induced ovulation in humans revealed the phenomenon of non-canonical meiosis, wherein the sister chromatid separation had occurred during meiosis I, and homologous chromosomes separated during meiosis II (Ottolini *et al.*, 2015). In this non-canonical mode of female meiosis, the secondary oocyte, which will now undergo meiosis I, is heterozygous. From this heterozygous genome, incoming sperm may preferentially choose one genome, and bias the fertilization outcome. Indeed, in wild mice populations, chromatid segregation in heterozygous female mice depends upon the genotype of the sperm; sperm with normal chromosome 1 favorably bias the outcome of fertilization in comparison to sperm that are abnormal for chromosome 1 (Agulnik *et al.*, 1993). These results can be explained in light of the recent discovery of reverse meiosis in human females (Ottolini *et al.*, 2015). Additionally, in canonical meiosis events, the sister chromatid which had undergone recombination during meiosis I is preferentially chosen as the oocyte genome over its sister which did not undergo recombination and becomes the second polar body (Ottolini *et al.*, 2015). Molecular mechanisms that govern non-canonical meiosis and the selective choice of recombined sister chromatid as the oocyte genome are not known. Additionally, how these dynamic variables during female meiosis allow oocytes and sperm to bias partner choices at fertilization, based on alleles that the gametes carry, is also unclear.

Physical interactions between egg and sperm at fertilization

After the correct sperm and egg encounter each other, the sperm must adhere to the egg and subsequently the egg and sperm cell membranes must fuse for successful entry of the male haploid genome into the female egg. The egg cortex is not homogenous in metazoans. In sea urchins, the surface of fertilization competent eggs is covered with microvilli which participate in fusion with the sperm membrane (Eddy and Shapiro, 1979; Longo *et al.*, 1994). Mammalian egg surface is covered with microvilli except in the region overlying the meiotic spindle, which is known as the amicrovillar region. Electron micrographs show that sperm heads fuse with the tips of the microvilli on the egg surface and sperm-egg fusion does not happen in the amicrovillar region (Yanagimachi, 1978). However, fertilization competent human eggs possess microvilli on their surfaces but do not have amicrovillar regions (Santella *et al.*, 1992). Thus, the role of amicrovillar regions on the mammalian egg membrane remains unclear. In mammals, a

member of the Tetraspanin family of proteins, CD9 localizes to the microvillar membrane on the eggs (Kaji *et al.*, 2000; Runge *et al.*, 2007). In mice, sperm cannot fuse with eggs that lack CD9 and loss of CD9 function in females renders the mice infertile (Kaji *et al.*, 2000; Le Naour *et al.*, 2000; Miyado *et al.*, 2000). Loss of CD9 from mammalian eggs alters the morphology of the microvilli on the egg surface, an effect that is also seen when eggs are exposed to Latrunculin, which inhibits actin (Runge *et al.*, 2007). Thus, it is inferred that microvillar morphology on the mammalian egg surface is critical in mediating fusion with the sperm and that this process relies on the actin cytoskeleton.

Biomembrane force probe experiments have attempted to understand physical properties of the egg membrane, which allow sperm-egg interaction to be maintained even when the sperm flagellum continues to move after binding. Such measurements reveal that the egg membrane of the amicrovillar region is comparatively stiffer than the microvillar region (Jegou *et al.*, 2008). Additionally, the egg membrane in both the microvillar and amicrovillar regions have elastic and viscoelastic properties which enable the extrusion of membrane tethers under forces similar to that exerted by flagellar movement of sperm bound to the egg (Jegou *et al.*, 2008). The viscous drag, combined with elastic deformation of the egg membrane tethers may transiently trap the sperm close to the egg so that downstream molecular events can be initiated.

After the sperm encounters eggs, the initial contact has to be consolidated into fusion of two completely different cellular membranes (Table 1). Izumo, a member of the Immunoglobulin super family is required on the sperm head membrane to fuse with mammalian eggs (Inoue *et al.*, 2005). Sperm from homozygous Izumo mutant male mice bound to the eggs, but could not fuse (Inoue *et al.*, 2005). Interestingly, recombinant Izumo protein binds mammalian eggs lacking CD9, indicating that CD9-Izumo interaction is not the primary determinant of egg-sperm fusion (Inoue *et al.*, 2013). The second strongest candidate on the egg surface that could interact with Izumo were Glycophosphatidylinositol (GPI)-anchored proteins because female mice in which GPI-anchored proteins were removed specifically from the eggs were infertile (Alfieri *et al.*, 2003). These initial observations culminated in the discovery of Juno, a GPI-anchored folate receptor on mammalian egg membranes as the cognate receptor for Izumo on mammalian sperm (Bianchi *et al.*, 2014; Chalbi *et al.*, 2014). Additionally, after egg-sperm fusion, Juno is rapidly lost from the egg membranes as extracellular vesicles in the perivitelline space (Bianchi *et al.*, 2014). The Juno shed from the egg membrane may potentially act as a decoy for additional acrosome-reacted sperm becuase Juno-Izumo interact with high affinities, providing a potential mechanism to prevent polyspermy in mammals (Aydin *et al.*, 2016). CD9, Izumo and Juno are conserved proteins and studies that elucidated its function in mammalian fertilization have shown cross-species functions. Thus it is unlikely that these three proteins mediate species specificity in mammalian egg-sperm fusion.

In vertebrates, the species specificity of egg-sperm fusion has only been experimentally proven in zebrafish, a fresh water minnow. Zebrafish eggs abundantly express a GPI-anchored Ly6/urokinase-type plasminogen activated receptor, aptly named as Bouncer (Herberg *et al.*, 2018). Interestingly, Bouncer homologs in external fertilizing species (fish and amphibians) are on the egg, while homologs in internal fertilizing species (reptiles and mammals) are on the sperm (Herberg *et al.,* 2018). Zebrafish females

that lack Bouncer produce eggs into which sperm cannot enter but Bouncer function is not required for formation of the micropyle, a specialized structure on teleost eggs that allow sperm entry (Herberg *et al.*, 2018). Thus, Bouncer functions in egg-sperm fusion after the sperm has entered the egg through the single micropyle on the teleost egg surface. Indeed intracytoplasmic injection of sperm into Bouncer deficient eggs resulted in successful fertilization and embryonic development (Herberg *et al.*, 2018). Expression of zebrafish Bouncer on the surface of the Medaka eggs resulted in zebrafish sperm being able to fuse with Medaka eggs, a cross-species fertilization event that otherwise never occurs (Herberg *et al.*, 2018). The species specificity of Bouncer in fertilization suggests that a dedicated partner must exist on teleost sperm, the identity of which is currently not known.

It is interesting that mammals posses highly specific molecular interaction such as Juno-Izumo during fertilization, but the interaction is promiscuous across species. It is tempting to speculate that this may have evolved because in internally fertilizing metazoans it is unlikely that gametes from different species would normally encounter each other. In externally fertilizing species such as fish, sea urchins and amphibians, gametes of different species may naturally encounter each other. In such conditions, proteins such as Bouncer in zebrafish and its homologs in other species ensure species-specific gamete fusion, with the help of a currently unknown sperm partner molecule.

Perspectives

Recognition, binding and fusion of egg and sperm of a species are existential requirements for survival of that species. A simplistic logic leads one to assume that when two cells, one of which is motile, needs to find its partner, external chemotactic cues would be advantageous. However, chemotactic behavior between sperm and egg can be promiscuous across species, suggesting that though useful, chemotaxis is unlikely to be the driving force for sperm-egg recognition. Yet, chemotactic behavior is a fundamental feature of egg-sperm interaction in all metazoans. Perhaps, chemotaxis is gametic bet hedging, to increase the odds of same species gamete finding each other (external fertilization) or to ensure that the fittest sperm reaches the egg (external and internal fertilization). It would be interesting to model or experimentally address gametic bet hedging by providing multiple chemoattractants to eggs and sperm of multiple species simultaneously. This would be insightful in externally fertilizing species, wherein such a scenario may naturally occur, for eg. different sea urchin species spawning in the same aquatic vicinity. A big unknown for most species is also the identity of the molecular partners on the egg and sperm that engage in binding and fusion. A significant advance would be if an egg-sperm membrane engaged in binding and/or fusion could be isolated specifically (for eg. by Click chemistry). Advancements in single cell transcriptome and proteome sequencing techniques could then identify the molecular partners involved in ensuring that gamete recognition culminates in binding/fusion. Finally, an aspect of fertilization that has received little to no attention from the perspectives discussed in this review is reproductive aging, commonly a result of changing hormone levels, especially in females. However, is the egg-sperm interaction itself subject to the influence of age and metabolic disorders? The identification of the molecular partners that ensure robust egg-sperm binding

and fusion should pave the way for addressing how this interaction changes with the age and metabolic state of the organism to alter the efficiency of fertilization.

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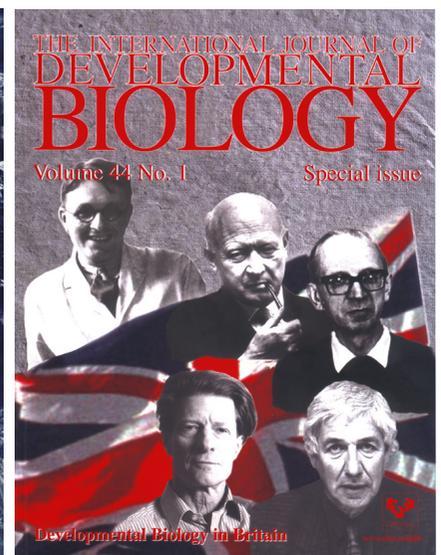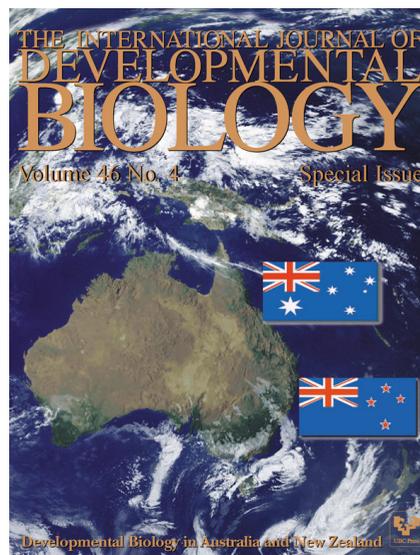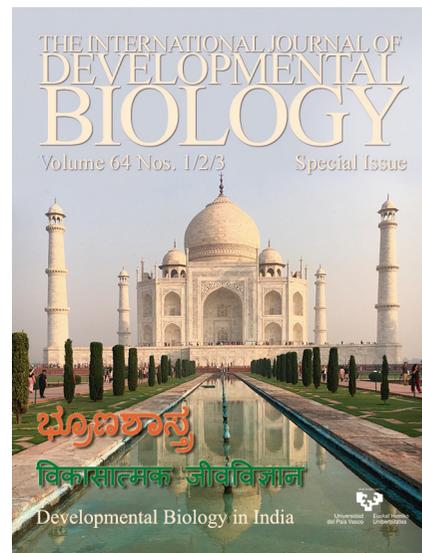
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