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INVITED REVIEWS AND SYNTHESES

Evolution of egg coats: linking molecular biology and ecology

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Abstract

One central goal of evolutionary biology is to explain how biological diversity emerges and is maintained in nature. Given the complexity of the phenotype and the multifaceted nature of inheritance, modern evolutionary ecological studies rely heavily on the use of molecular tools. Here, we show how molecular tools help to gain insight into the role of egg coats (i.e. the extracellular structures surrounding eggs and embryos) in evolutionary diversification. Egg coats are maternally derived structures that have many biological functions from mediating fertilization to protecting the embryo from environmental hazards. They show great molecular, structural and functional diversity across species, but intraspecific variability and the role of ecology in egg coat evolution have largely been overlooked. Given that much of the variation that influences egg coat function is ultimately determined by their molecular phenotype, cutting-edge molecular tools (e.g. proteomics, glycomics and transcriptomics), combined with functional assays, are needed for rigorous inferences on their evolutionary ecology. Here, we identify key research areas and highlight emerging molecular techniques that can increase our understanding of the role of egg coats in the evolution of biological diversity, from adaptation to speciation.

Keywords: diversification, egg coats, glycomics, natural selection, proteomics

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Introduction

One central goal of evolutionary biology is to explain how biological diversity emerges and is maintained in nature. However, due to the multifaceted levels of organismal diversity (from DNA sequences to complete phenotypes), and the complexity of mechanisms of inheritance (from direct genetic to epigenetic and parental effects) (Danchin *et al.* 2011), modern evolutionary ecological studies are increasingly reliant on molecular approaches, such as genomics (Hawkins *et al.* 2010) and proteomics (Diz *et al.* 2012). In this review, we highlight how combining molecular and ecological studies can help to understand the variability and the evolutionary role of egg coats.

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Egg coats are maternally derived extracellular structures that surround eggs and embryos, and consist of multiple functionally and structurally different layers (Box 1). Egg coats show great molecular, structural and functional diversity across species (Monne *et al.* 2006; Wong & Wessel 2006; Menkhorst & Selwood 2008; Box 1). They are important components of reproductive fitness as they mediate the beginning of life (due to their fundamental role in fertilization; reviewed in Monne *et al.* 2006; Wong & Wessel 2006; Menkhorst & Selwood 2008; Claw & Swanson 2012) and can affect embryonic performance by providing a dispersal and attachment medium and by protecting from biotic and abiotic hazards (Table 1). However, as we argue in this review – their broad evolutionary and ecological significance, as well as intraspecific variation, is currently underappreciated.

There are many reviews on the role of egg coats in sperm–egg interactions (Jovine *et al.* 2005; Wong & Wessel

Box 1. Basic structure and terminology of egg coats

For consistency, we designate the whole extracellular structure as 'egg coats' in our review. At their simplest, egg coats can be divided into oocyte coats (the innermost coats) and various kinds of outer coats (Box Fig. 1). In this review, we divide egg coats into three core types of structures: oocyte coats, jelly coats and postzygotic coats based on their timing of formation (i.e. before or after fertilization) and functions.

The oocyte coats include the protein-rich layers innermost (nearest to egg/embryo) of the different types of egg coats. The outer coats (i.e. those surrounding the oocyte coats) can vary from the sugar rich thin or thick gelatinous structures (i.e. jelly coat) of many molluscs, insects and amphibians, to the highly variable protein-rich egg capsules of marine invertebrates or highly mineralized egg shells of birds (Wong & Wessel 2006; Menkhorst & Selwood 2008). Different taxa have different combinations of these coats (e.g. only having the oocyte coat vs. having oocyte coat and a thick jelly coat vs. having all three types of coats; for good overviews, see Wong & Wessel 2006; Menkhorst & Selwood 2008). Finally, all of the egg coats can have differentiated layers, whereby the innermost layer (closest to the egg/embryo itself) of oocyte coats is called variably the vitelline envelope, zona pellucida or zona radiata (see notes on nomenclature). Also, the number and composition of jelly coats varies strongly even among related taxa (e.g. Altig & McDiarmid 2007). The postzygotic coats are typically the most complex and frequently consist of several highly differentiated layers (Wong & Wessel 2006; Menkhorst & Selwood 2008).

The oocyte coats and jelly coats are produced prior to fertilization, and both of them play roles in the fertilization process, whereby jelly coats may not be essential in fertilization and can have important ecological roles in many taxa (see main text). It is important to note that modifications occurring after fertilization are common in both oocyte coats and jelly coats (Wong & Wessel 2006). Consequently, the oocyte coats may be called 'vitelline envelope' prior to fertilization and 'fertilization envelope' after fertilization. Postzygotic coats are not involved in fertilization but can have important effects on embryonic performance (Menkhorst & Selwood 2008).

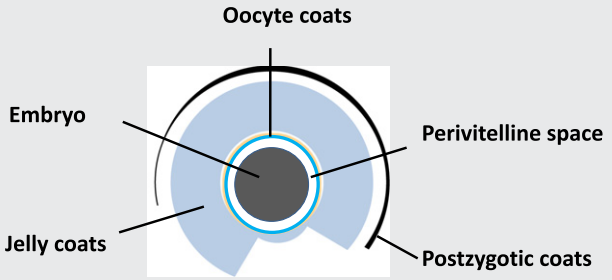
The site of egg coat production is variable: the oocyte coats originate usually during oogenesis from the oocyte or follicle cells, but sometimes in the liver (in some fish; Sano *et al.* 2013). The jelly coats are usually produced in the mothers' oviduct, liver or uterus, but sometimes in the follicle cells in the ovary (Wong & Wessel 2006). Postzygotic coats are produced by the mother in the oviduct, liver or uterus (Wong & Wessel 2006; Menkhorst & Selwood 2008) and sometimes, as in spiders, in specialized glands (e.g. Stubbs *et al.* 1992; Garb & Hayashi 2005).

Notes on nomenclature

The names used for different components of egg coats, as well as the genes coding for them, are highly variable across different research fields and different taxa, which easily result in confusion. For instance, the generic names can range from egg coats (used in this review) to extra cellular matrix, egg capsule and egg envelope to egg shell – the use of which is not taxa specific. Likewise, oocyte coat layers are named differently both between and within taxa. For instance, the innermost of the oocyte coats are called vitelline layer in echinoderms, vitelline membrane in dipterans, zona radiata or chorion in fish, vitelline envelope or zona radiata in amphibians, vitelline envelope or perivitelline membrane in birds and zona pellucida in mammals (Wong & Wessel 2006; Hedrick 2008; Claw & Swanson 2012). The nomenclature of egg coat glycoproteins is also confusing as they are named differently depending on the methods by which they were identified (Goudet *et al.* 2008). When analysed using SDS-PAGE, the glycoproteins are identified and generally named by their molecular weight (e.g. gp37, Kubo *et al.* 2000), whereas when they are analysed using cDNA cloning, they are named by gene name (e.g. ZPD, Lindsay *et al.* 2002). For classification of ZP subfamily genes of oocyte coats (see main text), we follow the recently defined nomenclature of six subfamilies: ZPA/ZP2, ZPB/ZP4, ZPC/ZP3, ZP1, ZPAX and ZPD (Wong & Wessel 2006; Goudet *et al.* 2008).

2006; Hedrick 2008; Menkhorst & Selwood 2008; Findlay & Swanson 2009; Claw & Swanson 2012; Gallo & Constantini 2012; Evans & Sherman 2013) as well as variation in structure and function among taxa (e.g. insects, Furneaux & Mackay 1972; marine inverbrates, Vacquier & Swanson 2011; fish, Rizzo *et al.* 2002; Berois *et al.* 2011; amphibians, Salthe 1963; Wake & Dickie 1998; Altig & McDiarmid 2007; eutherians, Denker 2000; Herrler & Beier 2000; in general: Wong & Wessel 2006; Menkhorst & Selwood 2008; Claw & Swanson 2012). As we will show, however, one major gap is apparent: egg coat-mediated natural selection that acts via embryonic performance is rarely considered in studies

Box 1. *Continued*



Box Fig. 1 A schematic presentation of the basic structure of the egg coats (as used in this review). The embryo is surrounded by a perivitelline space, oocyte coats (in all sexually reproducing taxa) and (taxa-dependent) jelly coats and/or various postzygotic coats. All coats can consist of multiple layers.

of egg coat evolution – particularly in the context of so-called oocyte coats and jelly coats (Box 1).

We argue that without considering this aspect of fitness, our understanding of the processes that influences the evolution of egg coats, and the genes mediating variability in them, may be hampered. We therefore emphasize the need for an integrative view that incorporates ecology (i.e. natural selection), the different roles of egg coats (i.e. sperm–egg interactions and embryonic performance) and intraspecific variation, from the molecular to the functional level, to gain insight into their role in evolutionary diversification (from adaptation to speciation). In this endeavour, the use of emerging molecular tools (i.e. glycomics, Varki *et al.* 2009; X-ray crystallography, Han *et al.* 2010; proteomics, Diz *et al.* 2012) is crucial, as most of the functional consequences of egg coats arise directly from their molecular phenotype (i.e. variation in their chemical and structural composition). When applying these tools to egg coats, we can quantify molecular variations and the functional consequences of these variations. This will then allow establishing the link between the genotype and the environment that determines egg coat composition, the contribution of egg coats to the early phenotype of an organism and, ultimately, the evolution of egg coats.

Why egg coats matter

Egg coats are indispensable structures that have multiple roles during an animal's early life stages and hence can have a strong impact on reproductive fitness. First, egg coats mediate fertilization – and hence the beginning of life itself – via sperm recognition, initiation of the acrosome reaction, sperm binding and blocking of polyspermy (e.g. Wong & Wessel 2006; Hedrick 2008). Second, egg coats have multiple roles during embryonic development. These range from providing a floating

Table 1 Selected set of examples for the diverse functional roles of egg coats. Different egg coat layers have partially different roles in these functions (see Box 1 and main text)

Function	References
Fertilization	
Increased sperm target size	Levitan & Irvine (2001), Podolsky (2002)
Sperm binding	Runft *et al.* (2002), Pang *et al.* (2011)
Acrosome reaction	Gunaratne (2007)
Block against polyspermy	Wong & Wessel (2006)
Species barrier	Turner & Hoekstra (2008a), Palumbi (2009), Hart *et al.* (2014)
Movement	
Dispersal	Goldberg *et al.* (2015)
Adhesion	Pechenik (1979), Rizzo *et al.* (2002)
Protection	
Oxygen transfer	Salthe (1963), Pinder & Friet (1994), Seymour (1994)
Thermoregulation	Salthe (1963)
Dehydration	Holmstrup & Westh (1995), Podrabsky *et al.* (2001)
Interaction with chemicals (e.g. salinity, pH, pollutants)	Pechenik (1982), Villalobos *et al.* (2000), Räsänen *et al.* (2003), Edginton *et al.* (2007), Rosa *et al.* (2015)
Pathogen resistance	Gomez-Mestre *et al.* (2006)
Acquirement of beneficial micro-organisms	Pinder & Friet (1994), Kerney *et al.* (2011)
Predation	Rawlings (1993), Roche *et al.* (2011)
Development	
Morphogenesis	Tsang *et al.* (2010)
Maternal signalling	Tadros & Lipshitz (2009)
Implantation	Marco-Jimenez *et al.* (2012)
Hatching	Gomez-Mestre *et al.* (2006), Touchon *et al.* (2006)

medium (in aquatic taxa) and medium for dispersal and attachment of eggs to the surroundings, to protecting the embryo from a range of abiotic (e.g. dehydration, UV radiation, salinity and pollutants) and biotic (e.g. predators and pathogens) environmental hazards (Table 1).

One important aspect of egg coats is that they are maternally derived (Box 1) (Menkhorst & Selwood 2008) and can therefore be an important source of maternal effects (Podolsky 2002; Räsänen *et al.* 2003; Räsänen & Kruuk 2007). Whereas egg size and egg content-related maternal effects are frequently addressed in animal studies (reviewed in Mousseau & Fox 1998), egg coats are rarely explored in this context. However, as egg coats often influence embryonic responses to environmental variation (Table 1), they can strongly influence individual reproductive success, determine responses of natural populations to environmental challenges and possibly influence the evolutionary trajectories of natural populations (e.g. Räsänen & Kruuk 2007). Finally, because of their joint role in sperm–egg interactions and their ecological importance, egg coats may act both as species barriers (e.g. Wong & Wessel 2006; Turner & Hoekstra 2008a; Palumbi 2009; Vacquier & Swanson 2011; Hart 2012) and be under natural selection (Podolsky 2002; Räsänen *et al.* 2003). Therefore, egg coats may contribute to both nonecological and ecological reproductive isolation (i.e. speciation, Coyne & Orr 2004; Palumbi 2009; Vacquier & Swanson 2011; Nosil 2012), a topic to which we will return below.

Despite this multitude of different functions, egg coats are still studied from a relatively narrow point of view and separately in different fields (e.g. molecular biology vs. ecology). Whereas molecular biologists and biochemists are typically interested in identifying molecules involved in sperm–egg interactions (e.g. Wong & Wessel 2006; Izquierdo-Rico *et al.* 2009; Pang *et al.* 2011) and medical researchers in linking their variation to pregnancy (e.g. Host *et al.* 2002; Assidi *et al.* 2015), ecologists are typically interested in their functional role and fitness consequences (e.g. Salthe 1963; Pechenik 1979; Podolsky 2002; Bovill *et al.* 2015). Here, we emphasize the need for integrative studies (combining molecular approaches with ecological studies) to gain insight into the multifarious role of egg coats and, in particular, the need to understand intraspecific variation (i.e. variation within and among populations of a given species) in them. We start by providing an overview of key aspects of interspecific variation in egg coats.

Interspecific variation of egg coats

Structure and function

Egg coats are complex extracellular structures (Box 1), present in all sexually reproducing animals, as well as many asexual metazoans (Wong & Wessel 2006). They can vary in size from a few microns (Wong & Wessel 2006; Menkhorst & Selwood 2008) to over 20 centimetres (e.g. Ebert & Davis 2007). In the following, we divide egg coats into three main types: prezygotic oocyte coats, prezygotic jelly coats and various postzygotic coats (Box 1). It is important to keep in mind that these different layers differ in their relative importance in sperm–egg interactions and embryonic performance and, hence, likely in the relative importance of sexual vs. natural selection (Box 1). Likewise, the relative length of time that offspring develop within these different structures (from a few hours to a few months) and, hence, their relative importance for embryonic fitness can vary strongly among taxa.

Oocyte coats

We term the innermost layers of the egg coats as oocyte coats (Box 1; Menkhorst & Selwood 2008). All sexually reproducing animals have oocyte coats and their basic functions are relatively similar across taxa: they have a key role in sperm–egg interactions and provide the basic protective layer to embryo (Wong & Wessel 2006). In most taxa studied to date, oocyte coats trigger the acrosome reaction in the sperm (Monne *et al.* 2006; Wong & Wessel 2006). In some taxa, only specific regions of the oocyte coat allow sperm entry (Wong & Wessel 2006). Moreover, some insects and most fish have one or more so-called micropyles, a special structure of the oocyte coat that attracts sperm and serves as a physical canal through which the sperm enters the egg (e.g. Amanze & Iyengar 1990; Yanagimachi *et al.* 2013). In addition, oocyte coats may contain sperm activation factors (e.g. reviewed in Wong & Wessel 2006; Cherr *et al.* 2008).

In general, oocyte coats are protein rich, consisting of fibrous structures formed of glycoproteins (Wong & Wessel 2006; Litscher & Wassarman 2007; Hedrick 2008; Wassarman *et al.* 2009; Claw & Swanson 2012). The molecular components of oocyte coats, particularly the zona pellucida (i.e. vitelline envelope), are well studied in many vertebrate, but only a few invertebrate, taxa (Wong & Wessel 2006). In vertebrates, oocyte coat glycoproteins normally share a common structural motif, known as the *zona pellucida* (ZP) domain (Jovine *et al.* 2005; Wong & Wessel 2006). ZP domain proteins have been found in egg coats of all vertebrate taxa studied to date (Wong & Wessel 2006; Wassarman *et al.* 2009) as well as some nonvertebrate taxa (e.g. gastropod molluscs, Monne *et al.* 2006; Aagaard *et al.* 2006, 2010; ascidians, Sawada *et al.* 2002; Yamada *et al.* 2009; branchiostoma, Xu *et al.* 2012). Within vertebrates, the basic structure of oocyte coats has been relatively conserved

during evolutionary history (Jovine *et al.* 2005; Monne *et al.* 2006; Wong & Wessel 2006; Litscher & Wassarman 2007). However, the composition of oocyte coats, as well as the genes coding for them, appears to be much more variable across invertebrate taxa (Wong & Wessel 2006). We return to this topic below when discussing egg coat genes.

Jelly coats

In many taxa (e.g. many insects, crustaceans, gastropod molluscs, echinoderms, fish and amphibians), oocyte coats are surrounded by so-called jelly coats (e.g. Salthe 1963; Segall & Lennarz 1979; Menkhorst & Selwood 2008; Box 1). Like oocyte coats, also jelly coats consist primarily of glycoproteins. However, the relative proportion of oligosaccharides (over proteins) is much higher than in the oocyte coats (e.g. Bonnell *et al.* 1994, 1996). For instance, in the African clawed frog (*Xenopus laevis)*, one of the model systems for jelly coat studies (Hedrick 2008), more than 60% of the jelly coats – but only approximately 10% of oocyte coats – consist of glycans (Yurewicz *et al.* 1975). These jelly coat glycans are highly variable and species specific, as seen in amphibians (e.g. Coppin *et al.* 1999a,b, 2003) and sea urchins (Segall & Lennarz 1979).

Jelly coats often also have a role in fertilization (Wong & Wessel 2006; Hedrick 2008). For instance, acrosome reaction-inducing substrate (ARIS) components have been identified in the starfish (*Asterias amurensis*) (Uno & Hoshi 1978), several other invertebrates (Naruse *et al.* 2011) and *Xenopus* (Ueda *et al.* 2003), and sperm activation factors can also occur in jelly coats (Wong & Wessel 2006). However, often the role of the jelly coat is primarily ecological: it provides an adhesive medium, interacts with the physical environment (e.g. mediating temperature and oxygen transfer; Salthe 1963; Pinder & Friet 1994; Seymour 1994) and provides protection against diverse environmental hazards (e.g. dehydration, Podrabsky *et al.* 2001; UV radiation, Marquis & Miaud 2008; predators, Roche *et al.* 2011; pathogens, Gomez-Mestre *et al.* 2006). Particularly in taxa which lack postzygotic coats (that cover many of the same ecological functions; see below), jelly coats should hence be prime targets for natural selection. Although glycobiology is entering studies of reproductive biology in relation to sperm–egg interactions (e.g. Claw & Swanson 2012; Gallo & Constantini 2012), the profiles as well as detailed functions, and the underlying genes, of jelly coat glycoproteins are still largely unknown. This is an important gap given the key biological functions (e.g. sperm–egg interactions and pathogen recognition) that glycans play in organismal cells (Varki *et al.* 2009). We dedicate our attention to this in particular in the section on strategies and molecular tools and Boxes 3 and 4.

Postzygotic coats

While oocyte coats and jelly coats are produced by the female *prior* to fertilization and act (at least in part) in fertilization, postzygotic coats are normally produced by the female *after* fertilization and have exclusively ecological functions (Menkhorst & Selwood 2008). Examples of postzygotic egg coats are the egg capsules in some marine invertebrates (e.g. Rawlings 1999; Westley & Benkendorff 2009), the ootheca in some insects and molluscs (e.g. Roth 1974; Nalepa & Lenz 2000; Goldberg *et al.* 2015), the cocoons of spiders (e.g. Stubbs *et al.* 1992; Garb & Hayashi 2005), the egg cases in some cartilaginous fish (e.g. Evans 1981; Heiden *et al.* 2005) and the egg shells of reptiles and birds (e.g. Hincke *et al.* 2012). Postzygotic coats can facilitate dispersal and/or attachment of the offspring, and protect the embryos from biotic (predators, parasites and pathogens) and various abiotic stressors (e.g. extreme temperatures, dehydration), while allowing gas and water exchange (reviewed in Menkhorst & Selwood 2008; Hincke *et al.* 2012).

Postzygotic coats often consist of many differentiated layers and are highly variable in shape, structure and composition among taxa (e.g. Menkhorst & Selwood 2008; Hincke *et al.* 2012; Goldberg *et al.* 2015). They usually consist of different kinds of proteins (such as collagen in shark egg cases: Evans 1981; or silk proteins in spider cocoons: Stubbs *et al.* 1992; Garb & Hayashi 2005), glycoproteins (e.g. egg capsules of marine invertebrates: Westley & Benkendorff 2009; Wasko *et al.* 2014) or calcium carbonate and other minerals (e.g. egg shells of birds; Hincke *et al.* 2012). In some model taxa, such as the domestic chicken (*Gallus gallus*; Hincke *et al.* 2012) and spiders (e.g. Garb & Hayashi 2005), the molecular and structural components as well as the corresponding genes of postzygotic coats have been identified. However, in general, relatively little is still known about variation in their molecular composition, their detailed functions and the underlying genes (Menkhorst & Selwood 2008; Hincke *et al.* 2012).

Evolution of egg coat genes

Based on phylogenetic analyses, the basic structure of oocyte coats appears relatively conserved across taxa – at least in vertebrates (Jovine *et al.* 2005; Claw & Swanson 2012). However, egg coat proteins can also evolve rapidly (Aagaard *et al.* 2006, 2013; Turner & Hoekstra 2008a; Findlay & Swanson 2009; Palumbi 2009; Vacquier & Swanson 2011). This dichotomy (conserved vs.

rapidly evolving) probably reflects the basic functions that egg coats need to assure, while being under strong and dynamic selective forces (via sperm–egg interactions and pathogens, in particular) (Findlay & Swanson 2009; Claw & Swanson 2012).

Oocyte coat genes

The genes coding for oocyte coats have been intensively studied in vertebrates (e.g. Claw & Swanson 2012; Meslin *et al.* 2012), but to a much lesser extent in invertebrates (Wong & Wessel 2006). In vertebrates, oocyte coat genes most commonly belong to the so-called *zona pellucida* domain (i.e. ZP genes; Wong & Wessel 2006; Wassarman 2008). The ZP gene family has thus far been reported in at least 74 species (GenBank), including all studied vertebrate taxa and some nonvertebrate taxa (e.g. ascidians, Sawada *et al.* 2002; gastropod molluscs, Monne *et al.* 2006; Aagaard *et al.* 2006, 2010; Yamada *et al.* 2009). ZP genes can be classified into six subfamilies and typically evolve through gene duplication and pseudogenization (Goudet *et al.* 2008; Claw & Swanson 2012; Meslin *et al.* 2012) (Fig. 1). Vertebrate taxa differ in the number and type of ZP genes, and the ZP3 gene is the only universal ZP gene (Fig. 1).

In vertebrates, the genetically best-understood egg coats are the matrices surrounding the ovulated eggs of the house mouse (*Mus musculus*). In the mouse, oocyte coats are coded by three ZP genes: mouse (m)ZP1, mZP2 (also called ZPA) and mZP3 (also called ZPC) (reviewed in Wassarman 2008; Claw & Swanson 2012). The suggested model for the mouse oocyte coat structure is a three-dimensional fibrous matrix, in which mZP2 and mZP3 form polymers that are cross-linked by mZP1 (Wassarman 2008). In contrast to the three ZP genes in the mouse, chicken has six, *X. laevis* five and humans four ZP genes (Goudet *et al.* 2008; Meslin *et al.* 2012). Classically, the mZP1 and mZP2 were deemed to be responsible for blocking polyspermy, whereas the mZP3 was assumed to be the sperm receptor and inducer of the acrosome reaction, in addition to being a structural protein. However, more recent work challenges the role of ZP3 in sperm-binding and indicates a role for ZP2 for sperm-binding in *Xenopus* (Tian *et al.* 1999), mice and humans (Avella *et al.* 2013, 2014).

The universal presence of the ZP3 gene in vertebrates is likely due to its fundamental role in oocyte coats, but it is also generally recognized as the ancestral gene of all other ZP gene families. The presence of pseudogenes indicates that several ZP genes have been lost during evolution (Goudet *et al.* 2008; Meslin *et al.* 2012). For example, the ZP4 occurs as a pseudogene in the mouse, while the ZP1 occurs as a pseudogene in dog, pig, cat and cow (Fig. 1). Most notably, the ZPD and ZPAX genes – which are present in *Xenopus* and chicken – have been pseudogenified or lost in all mammals (Goudet *et al.* 2008; Meslin *et al.* 2012) (Fig. 1). For fish, the phylogeny of ZP genes is less well resolved because of frequent gene duplications (Goudet *et al.* 2008; Meslin *et al.* 2012; Sano *et al.* 2013).

The reason for the loss of some ZP genes in mammals is currently not clear (Goudet *et al.* 2008), but may be due to differences in the selective environments that different taxa are exposed to (Wong & Wessel 2006). First,

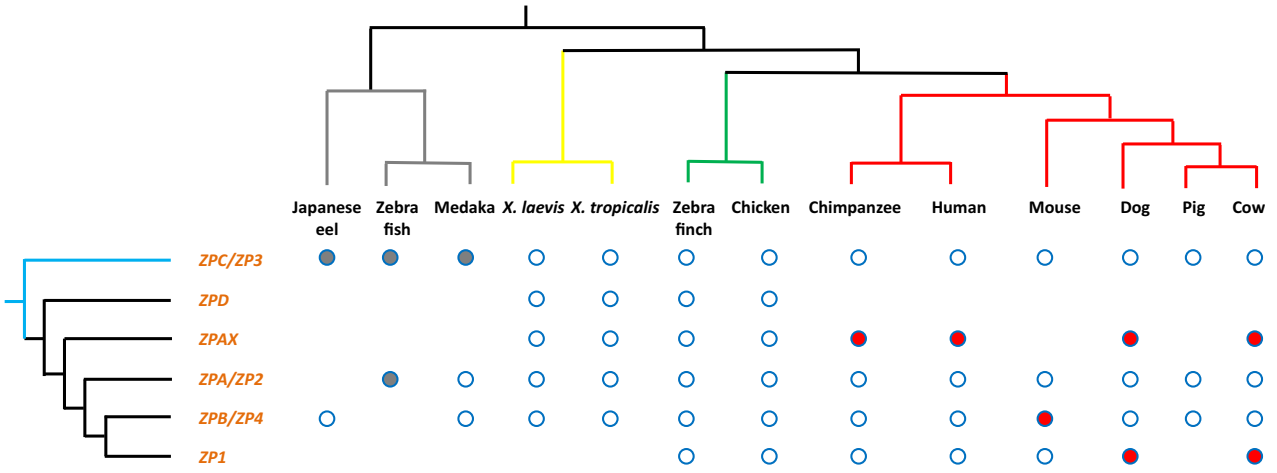


Fig. 1 A schematic illustration of the evolutionary loss and gain of ZP genes in major vertebrate groups. ZP gene subfamily names are given on the left and species names at the top (following Goudet *et al.* 2008) (see also Box 1). Circle: gene present; red circle: pseudogene (Goudet *et al.* 2008; Meslin *et al.* 2012); grey circle: gene duplication; blank: no gene exists (Table S1, Supporting information). Branch colours highlight fish (grey), amphibians (yellow), birds (green) and mammals (red). The blue line in the evolution of the ZP gene family indicates the unclear evolutionary origins of the ancestral ZPC/ZP3 gene (Goudet *et al.* 2008; Claw & Swanson 2012). The genes used here originate from GenBank (see Table S1, Supporting information).

the loss of genes may relate to shifts from external to internal fertilization, although phylogenetic studies do not provide unambiguous support for this hypothesis (Goudet *et al.* 2008). Alternatively, it is possible that some ZP genes are lost in mammals because they do not play a significant role in matrix formation and sperm–egg interactions (Goudet *et al.* 2008; Meslin *et al.* 2012). Second, given that egg coats have important ecological functions, it is also possible that the evolution of ZP genes is influenced by natural selection acting via embryonic performance. For instance, in organisms with external development, such as most fish and amphibians, and birds, embryos develop in risky environments and, hence, often require more additional functions (see Table 1) from egg coats than do taxa with internal development. It may therefore be that in taxa with internal development, the genes coding for these additional structures/functions are present only as pseudogenes (Wong & Wessel 2006; Goudet *et al.* 2008). However, the role of ecology in the evolution of ZP genes, as for egg coat genes in general (Jagadeeshan & Singh 2007), seems to have been largely ignored in current empirical work and, hence, further studies are needed to test this hypothesis. Moreover, as ZP domain proteins also function outside egg coats (Jovine *et al.* 2005), the evolution of ZP genes is likely to be affected also by egg coat-independent processes (Wong & Wessel 2006).

In invertebrates, the oocyte coat genes have been mainly studied in marine invertebrates, particularly sea urchin and abalone (reviewed in Vacquier & Swanson 2011), and a small number of insects (fruit flies, Jagadeeshan & Singh 2007; Gonçalves *et al.* 2013; lepidopterans, Carter *et al.* 2013; mosquitoes, Marinotti *et al.* 2014). *Drosophila* and the silk moth (*Bombus mori*) are classical model systems for the developmental genetics of oocyte coats (reviewed in Papantonis *et al.* 2015).

In contrast to vertebrates where ZP genes are universal, the genes coding for invertebrate oocyte coats do not appear to be as conserved across taxa (Wong & Wessel 2006). First, the ZP domain has been found in the oocyte coats of marine invertebrates (see above), whereas several different oocyte coat genes are found in other invertebrates. Examples of these are the EBR1 and rendezvin in sea urchins (Wong & Wessel 2006; Vacquier & Swanson 2011), different Vitelline membrane protein (VMPs) and chorion genes in *Drosophila* (Jagadeeshan & Singh 2007; Papantonis *et al.* 2015), the silk moth (Papantonis *et al.* 2015) and other lepidopterans (Carter *et al.* 2013), mosquitoes (Marinotti *et al.* 2014), and the Brownie and Citrus genes in the cockroach *Blattella germanica* (Irles *et al.* 2009; Irles & Paliuchi 2011). The functions of these genes vary from structural components, sperm–egg interactions to embryonic protection, and existing evidence suggests that, despite basic conserved structure of the oocyte coats, the evolution of animal egg coats can be highly dynamic (Wong & Wessel 2006; Claw & Swanson 2012). Further studies on a much broader range of animal taxa are needed, however, to have a good understanding of the evolution of oocyte coat genes.

Jelly coat and postzygotic coat genes

There is currently a relative scarcity of data for genes coding for jelly coats and postzygotic coats. The few studies available for jelly coat genes have focused on ARIS genes: three ARIS genes (ARIS1, 2 and 3) have been reported in starfish (*Asterias amurensis*) (Uno & Hoshi 1978) and several other echinodermata (Naruse *et al.* 2011), while the ARISX gene has been found for *Xenopus* jelly coats (Ueda *et al.* 2003). It has been proposed that the sugar chain of the ARIS molecule could provide the variation needed for species-specific egg–sperm recognition, whereas the protein component may maintain the basal conserved structure (Naruse *et al.* 2011). These findings support the role of jelly coats in fertilization, but the genes coding for the remaining components of jelly coats, and their ecological roles, are largely unknown (Box 3).

With regard to genes coding for postzygotic coats, among the best studied are the avian egg shells in the domestic chicken and the zebra finch (Hincke *et al.* 2012), and the cocoons of spiders (e.g. Garb & Hayashi 2005; Starrett *et al.* 2012). In birds, a large number of different genes code for egg shells (reviewed in Hincke *et al.* 2012) – not surprising given the complex structure of avian eggs. In spiders, several spidroin and egg case protein (ECP) genes code for the egg cases (e.g. Garb & Hayashi 2005; Starrett *et al.* 2012). However, both for jelly coats and for postzygotic coats, only a handful of species have been studied thus far. This emphasizes the need for more molecular genetic studies to allow rigorous insight into the evolutionary ecology of jelly and postzygotic coats.

The missing component: intraspecific variation

One of the core points of our review is to highlight the importance of *intra*specific variation in egg coats. Why do we care about intraspecific variation in them? As for any other trait, intraspecific phenotypic variation is the raw material for selection to act upon, reflects the selective history and the potential of natural populations to evolve in response to environmental change. Furthermore, when studying egg coat variation at early stages of divergence, we can gain insight into the mechanisms facilitating speciation (e.g. Turner & Hoekstra 2008a; Nosil 2012).

Most of the data to date on selection on egg coats comes from DNA sequence-based analyses on oocyte

coats (e.g. *Drosophila*, Jagadeeshan & Singh 2007; mammals, Turner & Hoekstra 2008b; sea urchins, Pujolar & Pogson 2011; Vacquier & Swanson 2011; abalones, Aagaard *et al.* 2013). Here, one of the best-characterized cases are the sperm receptors (VERL and VEZP) in the oocyte coats of abalones, which are responsible for gamete interaction and essential during fertilization (Box 2; Galindo *et al.* 2002; Aagaard *et al.* 2006, 2010, 2013). With regard to jelly coats, intraspecific variation in fertilization success has been found in relation to variation in jelly thickness in the echinoid *Dendraster excentricus* (Levitan & Irvine 2001; Podolsky 2001) and in the effects of jelly on sperm motility in the frog *Crinia georgiana* (Simmons *et al.* 2009). With regard to intraspecific variation in the molecular composition of jelly coats, very little is known. One of the very few studies was carried out on jelly coat mucins in *X. laevis* (Guerardel *et al.* 2000), where intraspecific polymorphism in O-glycans equivalent to that of human blood groups was found. However, this polymorphism had no consequences for fertilization success (Guerardel *et al.* 2000), indicating that this jelly coat variation may not be relevant for sperm–egg interactions.

A role of egg coats in adaptive divergence and speciation?

Few studies have directly quantified intraspecific variation of egg coats at the phenotypic and functional level – and the consequences of this variation for diversification of natural populations. As stated above, this is a clear gap because egg coats are a prime source of adaptive maternal effects, with potential to influence evolutionary responses at ecological timescales (Räsänen & Kruuk 2007). An example of egg coat-mediated adaptive divergence – and intraspecific variation in jelly coats – comes from our own work on two ranid frogs, where among-population divergence in embryonic acid stress tolerance is mediated via jelly coats (Box 4). Although this work is only part way to understanding the molecular basis of egg coat-mediated adaptive maternal effects (Box 4), the data demonstrate the high level of intraspecific variation in jelly coat composition (based on SDS-PAGE analyses, measurements of jelly coat zeta potential and water balance; Box 4). Evidence for intraspecific variation for postzygotic coats comes from recent studies on geographic variation in egg shell structure of the house finch *Carpodacus mexicanus* (Stein & Badyaev 2011) and the pied flycatcher (*Ficedula hypoleuca*) (Morales *et al.* 2013), although the genetic basis of this variation was not established. Studies on a broader range of taxa and in relation to other putative selective factors are very much needed to shed light on the role of egg coats in adaptation.

Egg coats can evolve rapidly and are an essential component of reproductive isolation (Palumbi 2009). Their role in sperm–egg interactions has long been extensively studied as a species barrier (e.g. Wong & Wessel 2006) and, subsequently, in speciation (Coyne & Orr 2004; Palumbi 2009; Vacquier & Swanson 2011; Hart 2012). Yet the integration of ecology in this process has been little considered to date. Here, the dual role of egg coats in ecologically relevant functions (i.e. environment-dependent effects on embryonic performance) and in sperm–egg interactions is of key importance. Along these lines, natural selection can result in faster evolution of egg coat layers that are of ecological importance compared to layers that have primary functions in sperm–egg interactions, as indicated for chorion vs. vitelline membrane genes in *Drosophila* (Jagadeeshan & Singh 2007).

If there is strong divergent natural selection on egg coats (e.g. different selective environments may favour different molecular composition of egg coats due to their impact on embryonic performance; Box 4), this might facilitate the evolution of reproductive isolation via adaptive divergence (i.e. ecological speciation, Turner & Hoekstra 2008a; Nosil 2012). Moreover, also sperm may be under divergent natural selection (e.g. Manier & Palumbi 2008; Byrne *et al.* 2015). Gene flow among populations could then be reduced either via direct viability selection against immigrants (Nosil *et al.* 2005; Hangartner *et al.* 2012) – mediated by differential embryonic performance – or due to the disruption of locally adapted (sperm and egg) genotype combinations (Findlay & Swanson 2009; Nosil 2012). On the other hand, the fundamental role of egg coats in fertilization may impose constrains on their continued evolution under natural selection if sperm–egg interactions are influenced by different selective forces acting on sperm and on the egg coats (Findlay & Swanson 2009; Palumbi 2009; Aagaard *et al.* 2013). Although there is some evidence for co-evolution of sperm–egg coat proteins in relation to fertilization (e.g. Clark *et al.* 2009), studies on how adaptive divergence in response to divergent natural selection may influence co-evolution of egg coats and sperm are, to our knowledge, currently missing.

A rare example linking intraspecific variation in egg coats with speciation comes from a recent study on the sea star (*Patiria miniata*), which showed that sperm–egg interactions have facilitated speciation between two clades (Hart *et al.* 2014). In this study, population differences in sperm–egg compatibility where investigated through fertilization experiments, whereby males and females from a southern and a northern *P. miniata* population were reciprocally crossed. RNA-seq methods were used to characterize coding sequence variation in relevant egg coat genes of *P. miniata*. Finally, combined analyses of fertilization success and molecular genetic population divergence in both sperm surface (binding)

Box 2. How to apply proteomics to the study of egg coats in nonmodel species

Proteomics has only recently rigorously entered the general domain of evolutionary ecology (Diz *et al.* 2012), but has been long part of egg coat studies (e.g. Yurewicz *et al.* 1975). The basic principle here is that the whole organism or tissue (e.g. oocyte or postzygotic coat) of interest is specifically analysed for its protein composition. This allows then identifying the proteins of interest and provides a link between the phenotype and the genotype (Diz *et al.* 2012). In the case of egg coats, proteomics is particularly relevant for the analyses of the protein-rich oocyte coats as well as several types of postzygotic coats (see main text). When aiming to understand the evolutionary ecology of egg coats, proteomics (and glycomics; Box 3) analyses are best combined with functional analyses of different egg coat variants and molecular genetic approaches (see main text; see also Findlay & Swanson 2009 for an overview of proteomics methods in studies of reproductive proteins). The approaches selected for functional analyses will of course depend on the question of interest (e.g. be it on sperm–egg interactions, effects of jelly coats on gas or ion exchange or pathogen defence, Table 1 main text).

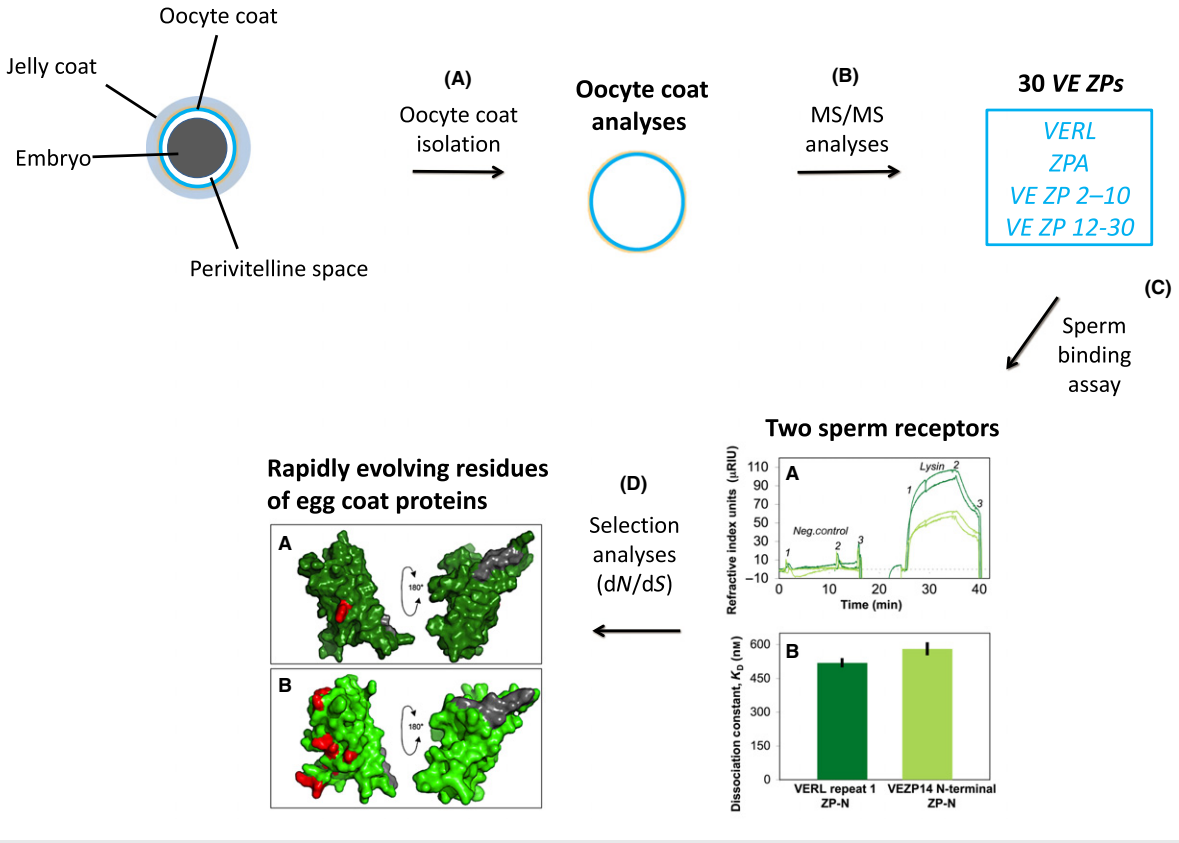
With regard to the analyses of egg coat composition and function, a few points of practical nature are important. First, when collecting egg coats for analyses, it is important to isolate the appropriate layer of interest (e.g. oocyte coat, jelly coat or a specific postzygotic coat) – while taking care to avoid contamination from components of the egg/embryo or other egg coat layers. To avoid biased inferences in molecular analyses of egg coats, egg/embryo components are hence often analysed as a reference (e.g. Xu *et al.* 2012). Second, as the prezygotic coats often undergo modifications upon fertilization (Box 1), functional importance of oocyte or jelly coats may best be analysed *before/during* fertilization if the interest is in sperm–egg interaction and *after* fertilization if the interest is in effects on embryonic performance. Third, because egg coat production is often transient (e.g. bound to reproductive seasons or to daily cycles), analyses aiming to identify genes or variation in gene expression need to collect the tissue of relevance (e.g. oocyte, oviduct) at the appropriate time of the reproductive cycle.

Studies in abalone provide a good example of how to apply a combination of proteomics, functional assays and selection analyses on questions related to sperm–egg coat evolution (Box Fig. 2). Proteomics analyses identified a high diversity of VE proteins with ZP domains in abalone (30 ZP proteins; Aagaard *et al.* 2006, 2010), providing general evidence that ZP proteins are constituents of the oocyte coats in marine invertebrates (Aagaard *et al.* 2006, 2010). Sperm-binding assays indicated that two vitelline coat receptors (VEZP 14 and VERL) are able to bind sperm (Galindo *et al.* 2002; Aagaard *et al.* 2013), and molecular genetic selection *analyses* (dN/dS) further suggest that both VERL and VEZP14 evolve rapidly. Structural models showed that sperm-binding receptors occur at the same face on the surface of both VERL and VEZP14 proteins (A: VERL and B: VEZP14, Aagaard *et al.* 2010, Aagaard *et al.* 2013).

Currently, one of the big remaining challenges in applying –omics approaches, in general, is the availability of databases. This is particularly true when working on nonmodel species for which genomes have not yet been sequenced – as is the case for most taxa of interest in evolutionary ecology. As a comprehensive database is essential for a successful proteomics project (Diz *et al.* 2012), the most detailed studies on egg coat proteins to date have been performed in model species with a sequenced genome. However, the field is developing fast and there are now several methods to overcome this challenge.

The most common approach is to use a reference genome(s) of one or more model species (Forne *et al.* 2010). Unfortunately, this method only works well on homologous proteins and depends on the phylogenetic distance between the reference and the target study species (Diz *et al.* 2012). Another approach is to use *de novo* sequencing. This technique can overcome the 'lack-of-database' problem, because it can infer the polypeptide sequences needed to identify and characterize proteins directly from the MS/MS spectra without the help of a sequence database (Dancik *et al.* 1999; Savitski *et al.* 2005). Finally, RNA-seq-based approaches are becoming available. These approaches can identify and quantify the transcriptome from both model and nonmodel species (Hawkins *et al.* 2010; De Wit *et al.* 2012), and the full-length cDNA library can subsequently be translated into protein sequences and used as a database in the MS data alignment (Wang *et al.* 2009). Knowledge on the site of egg coat production (e.g. follicle oocyte vs. liver vs. oviduct) will then allow tissue-specific transcriptomic analyses (see Hart 2012 for example on sperm and egg coat proteins in marine invertebrates and Hincke *et al.* 2012 for an example on bird egg shells).

Box 2. *Continued*



Box Fig. 2 A schematic representation of studies of egg coat proteins in abalone (*Haliotis* spp.). In abalone (marine gastropod molluscs), oocyte coats have important functions in fertilization and contribute to the rapid evolution of reproductive isolation among abalone species (Aagaard *et al.* 2013). Abalone eggs are surrounded by the perivitelline space, a vitelline envelope (VE; i.e. the oocyte coat) and a fibrous jelly coat. The figure shows the key steps used to investigate the constituent proteins of the oocyte coats and to study the evolution of reproductive proteins. A) VEs were isolated and solubilized, B) VE glycoproteins were identified using a series of SDS-PAGE and MS/MS analyses, C) in vitro sperm-binding assays were conducted to test for physical interactions between a sperm protein (lysin) and two sperm receptors and D) molecular genetic selection analyses where conducted on ovary expressed genes to test for co-evolution between the sperm proteins and sperm-binding VE components. Finally, structural models were used to map positively selected residues (grey fills in the 3D structures) on surface of egg coat proteins. (Figures C and D are adapted from Aagaard *et al.* 2013 with the Open-Access License).

and egg coat (OBi1) genes indicated that the putative driver of jelly coat diversification in this system is sexual conflict (Hart *et al.* 2014). However, ecologically mediated divergence (i.e. via population density-related sperm competition) remained an alternative explanation.

Egg coats, particularly oocyte and jelly coats, are simultaneously under many different selective forces (i.e. sperm–egg interactions, interactions with the external environment and pathogens), which necessitate dynamic responses to external conditions. Such a need for multifactorial responses to divergent selection poses challenges for their evolution, as well as inferring the genetic basis and functional consequences of their molecular variation (Box 3). In addition, egg coat-mediated adaptation may require co-evolution with genes, such as the hatching enzyme, expressed in the embryo itself (e.g. Lepage & Gache 1990; Kawaguchi *et al.* 2007). Hence, studies considering the ecological functions of egg coats would increase our understanding of co-evolution of sperm–egg coat proteins and maternal–nuclear genes and, ultimately, of to what degree egg coats contribute to adaptation and the evolution of ecological reproductive isolation.

Research gaps and challenges

Although a number of studies have been carried out to identify the structural and molecular genetic bases of egg

Box 3. Egg coat glycomics

Glycans are often a key component of various egg coats, in particular the jelly coats. In general, glycans perform key biological functions in organismal cells (Varki *et al.* 2009) and represent dynamic and complex structures with variable amounts and types of saccharides, arranged in multiple branches (antenna). In glycoproteins, glycan structures are attached to a protein backbone (Box Fig. 3). The biosynthesis of a given glycan can involve several different enzymes, and in contrast to proteins, there is no direct template between the DNA sequence and the final glycan structure. Due to the large variability in the glycan structures, variation in their biosynthesis pathways and often large number of genes involved, they are challenging to analyse (Varki *et al.* 2009; Bennett *et al.* 2012; Joshi *et al.* 2015; Nairn & Moremen 2015). As the analytical details are beyond the focus of this review, we here shortly summarize the key points and advice the reader to consult recent empirical work on egg coats (e.g. Li *et al.* 2011) and generic reviews for glycan analyses (Varki *et al.* 2009; Jensen *et al.* 2010, 2012; Bennett *et al.* 2012; Joshi *et al.* 2015) and the emerging field of glycotranscriptomics (Nairn & Moremen 2015).

The two major types of oligosaccharides are N-linked and O-linked glycans, named according to their point of attachment to proteins (Varki *et al.* 2009; Jensen *et al.* 2010, 2012). Typically, N-linked glycans are a common feature of the oocyte coats, whereas O-linked glycans are a major component of jelly coats (Wong & Wessel 2006; Hedrick 2008). The basic steps of glycan analyses from egg coat glycoproteins may include first (as in proteomics) 1D or 2D gel-based analyses to establish macromolecular variation. For subsequent analyses of glycans (either from the composite gel or from isolated bands of interest), the glycans are detached from their protein backbone and their amount and structural variability analysed (Box Fig. 3). For analytical purposes, N-linked glycans can be detached from their protein backbone by specific enzymes, such as PNGase F (Jensen *et al.* 2010, 2012). In contrast, there is no universal enzyme for O-glycan release, and therefore, chemical methods (e.g. β-elimination) are often used when analysing O-linked glycans (Jensen *et al.* 2010, 2012).

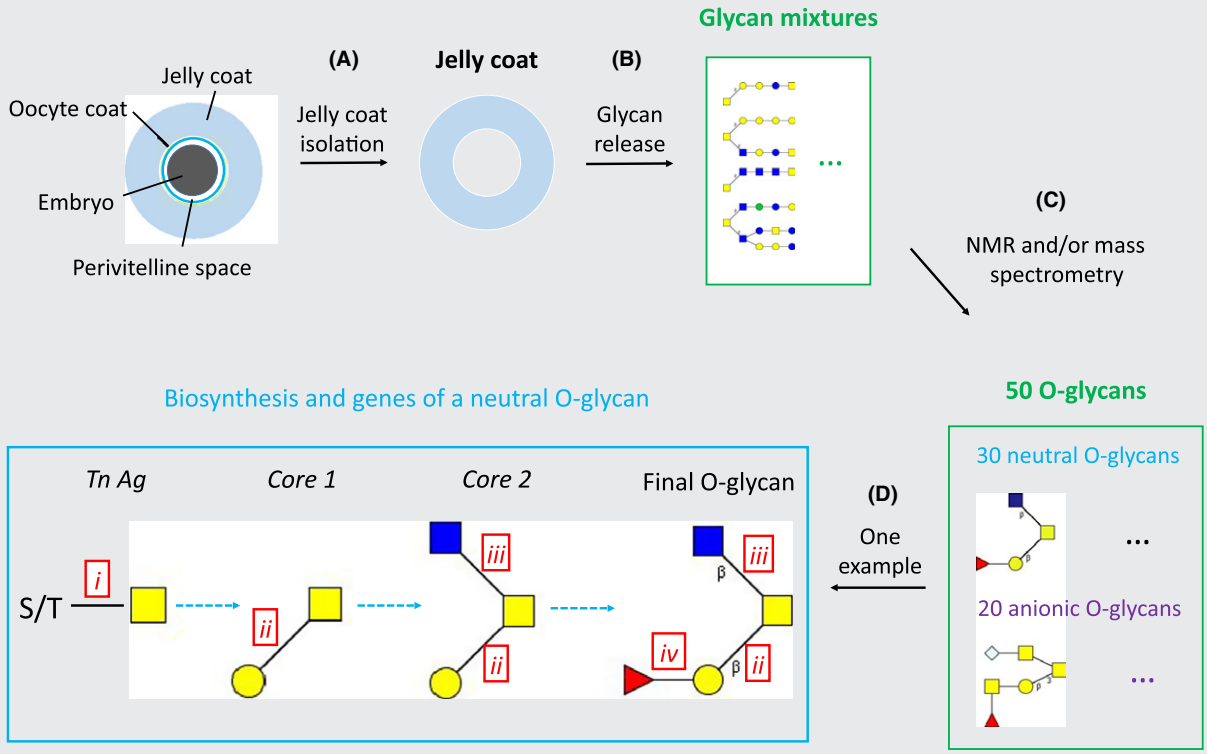
Following chromatographic separation, structural analysis of glycans is usually performed by NMR or MS. NMR spectroscopy is a powerful tool for *de novo* structural characterization (Lundborg & Widmalm 2011), while MS-based methods can perform high-throughput glycomic profiling (Jensen *et al.* 2010, 2012). Once detailed structural information of glycans is acquired, the underlying genes and biosynthesis pathways can be identified, thus allowing inference of the genetic basis of glycan variation (Nairn *et al.* 2008; Varki *et al.* 2009; Campbell *et al.* 2014; Nairn & Moremen 2015).

Jelly coat glycoproteins consist largely of so-called mucin-type O-glycans, which are particularly challenging to study: they show extreme variability in structure, often a large number of genes are involved in their biosynthesis and they show differential expression (reviewed in Jensen *et al.* 2010; Bennett *et al.* 2012). Although jelly coat glycan composition has been quantified in several species, empirical examples for *intra*specific variation in jelly coats are sparse. As an example of integrative approaches to study jelly coat variation in the domain of evolutionary ecology, we present an overview on our own studies on amphibians, where jelly coats contribute to adaptive divergence along an acidification gradient (Box 4).

General methodological considerations

In undertaking analyses of egg coat glycans, many of the same methodological considerations apply as in proteomics (Diz *et al.* 2012; Box 2). Here, careful sample preparation (e.g. isolation of relevant egg coats and avoiding contamination from embryonic proteins) and sufficient level of biological and technical replication are essential. In contrast to the relatively simple and cheap gel-based analyses (which can usually be easily undertaken in-house as the first step to quantify macromolecular variation of oocyte or jelly coats), in-depth analyses of glycans are technically challenging and require access to specialized equipment. Outsourcing of glycan analyses has recently become available, but is currently rather costly. The most challenging issue arises, however, from the high complexity of the glycan biosynthesis (especially of jelly coats) – and the simultaneously acting different selective forces (e.g. abiotic stress, pathogens and sperm–egg interactions). These pose a challenge for identifying the link between the molecular composition, the functional consequences and the genetic basis of this variation (Nairn *et al.* 2008; Bennett *et al.* 2012; Joshi *et al.* 2015; Nairn & Moremen 2015). Hence, a good study design and expertise in glycan bioinformatic analyses are needed.

Box 3. *Continued*



Box Fig. 3. A schematic representation of the key steps for investigating the structure and the biosynthesis pathway of egg coat glycans as applied to jelly coats of *Xenopus laevis*. In *X. laevis*, eggs are surrounded by a perivitelline space, the oocyte coat and a jelly coat. The jelly coat consists primarily of mucin-type O-linked glycans. To analyse glycan structure, A) the jelly coat is isolated and solubilized, B) O-glycans are released through β-elimination, C) following chromatographic separation, structural analysis of glycans is performed by NMR (Strecker *et al.* 1995; Guerardel *et al.* 2000) or MS (Xie *et al.* 2004). These approaches have identified a total of 50 O-glycans, including 30 neutral O-glycans and 20 anionic glycans in X. laevis (Strecker *et al.* 1995; Guerardel *et al.* 2000; Xie *et al.* 2004).
In D), the complexity of the glycan biosynthesis pathway is exemplified for one *Xenopus* jelly coat mucin-type O-glycan (Fuc(a1-2)Gal(β1-3)[GlcNAc(β1-6)]GalNAc-ol; indentified in Guerardel *et al.* 2000). In the initiation step of the biosynthesis of a mucin-type O-glycan (Varki *et al.* 2009; Bennett *et al.* 2012), a monosaccharide called N-acetylgalactosamine (GalNAc, yellow square) is added to the protein backbone (either to a serine (S) or threonine (T) residue), which forms an antigen (here Tn Ag). Subsequently, different monosaccharides (indicated by the different colours and shapes) are sequentially added. These then form different core structures (here Core 1 and Core 2) and, finally, the final O-glycan. The enzymes responsible for the different steps are coded by different genes. As the mucin-type O-glycan biosynthesis pathway is well established in *Xenopus*, the genes related to the biosynthesis of known glycans can be inferred from the KEGG database (Kanehisa & Goto 2000). In this example, the final glycan structure (Fuc(a1-2)Gal(β1-3)[GlcNAc(β1-6)]GalNAc-ol) is the product of at least four different genes (i = polypeptide N-acetylgalactosaminyltransferase; ii = β1, 3-galactosyltranseferase; iii = β1, 6-N-acetylglucosaminyltransferase and iv = a1,3-fucosyltransferase).

coat variations, important questions to be addressed include the following: (i) How much intraspecific variation do egg coats harbour? (ii) What is the functional role of the molecular variation of egg coats? (iii) What role do egg coats play in ecological and evolutionary processes of natural populations?

Given the fundamental role of egg coats in reproductive success, the above questions are important in evolutionary ecology. However, they are difficult to answer without the use of modern molecular tools and more integrative approaches in order to understand the links between intraspecific variability (which is often only apparent when using molecular approaches), structure and function. First, intraspecific variability of egg coats has to date been almost exclusively studied at the DNA sequence level – rather than at the phenotypic level. Yet, it is the phenotype (including its genetic and plastic components) that expresses the function and is the direct target of natural selection, at least in the short term (Houle *et al.* 2010). Therefore, DNA sequence variation alone may not be sufficient for understanding evolutionary processes (Houle *et al.* 2010; Danchin *et al.* 2011). The

Box 4. Divergent natural selection on jelly coats along an acidification gradient

Two closely related ranid amphibians, the moor frog (*Rana arvalis*; RA) and the common frog (*R. temporaria*; RT), breed in ponds that range from acidic (pH 4–4.9) to neutral (pH 7) along an acidification gradient in SW Sweden (Hangartner *et al.* 2012). In amphibians, embryos often show high mortality at acidic pH (reviewed in Räsänen & Green 2009), indicating potential for strong acidity-mediated natural selection. This high embryonic mortality at acidic pHs classically has been assigned to the so-called curling defect (reviewed in Räsänen & Green 2009), whereby embryos can develop but fail to hatch or hatch in abnormal shape (i.e. due to tight coiling inside egg coats). Jelly removal experiments have shown that this curling defect is, at least in part, dependent on the maternally derived gelatinous egg coats (reviewed in Räsänen & Green 2009) (Box Fig. 4A).

There is evidence for adaptive divergence in embryonic acid tolerance within both RA and RT (reviewed in Räsänen & Green 2009; Brunold 2009). To infer the mechanisms underlying adaptive divergence, we have applied a combination of experimental and molecular tools. First, reciprocal crosses among populations, combined with jelly removal experiments and common garden rearing of embryos at different pHs, revealed that variation between and within populations in embryonic acid tolerance is determined by jelly coat-related maternal effects (Brunold 2009; Hangartner *et al.* 2012; reviewed in Räsänen & Green 2009). Second, in RA, analyses of water retention of the jelly coats show that jelly coats lose water under acidic pH, but that this water loss is reduced in jelly coats from an acid-adapted population (Shu *et al.* 2015). Third, electric charge measurements (indicative of glycosylation status of jelly coats, Shu *et al.* 2015) indicate that glycan composition of jelly coats correlates with jelly water balance as well as with embryonic acid tolerance. Fourth, SDS-PAGE analyses, combined with multivariate statistics, show extensive macromolecular variation in the composite jelly coats (Box Fig. 4B) and that this variation is associated with differences in embryonic acid stress tolerance within both species (Shu 2014).

Fifth, NMR spectroscopy and different mass spectrometric approaches have been applied to both oviduct (the site of jelly coat glycan biosynthesis in amphibians) and jelly coat glycans. Oviduct glycan composition of RA and RT is highly diverse, but species specific: 19 different glycans have thus far been identified in RA and 13 in RT (Coppin *et al.* 1999a,b). To gain first insight into potential for *intra*specific variation in jelly coat glycans, we recently conducted mass spectrometric analyses commercially from jelly coats of 10 RA females. These indicate a high diversity in jelly coat glycans among individuals (L. Shu, M. J.-F. Suter & K. Räsänen, unpublished), but to what extent this variation is related to embryonic acid tolerance is currently under study.

Ultimately, studies on egg coat-mediated evolutionary processes (such as ours) will want to investigate the genetic basis of egg coat variation. This poses a clear challenge in nonmodel taxa, such as RA and RT. First, no genome is available from closely related species (the only amphibian for which the complete genome is available is *X. tropicalis*, which diverged from ranids approximately 200 Ma). Second, as the mucin-type O-linked glycans of jelly coats probably reflect responses to many different factors simultaneously (Bennett *et al.* 2012), a combination of structural identification of glycans (see above Box Fig. 3) and transcriptomics (Nairn & Moremen 2015) is needed to study jelly coat evolution. As a first step towards establishing a genetic basis of jelly coat variation, we applied *de novo* transcriptomics (RNAseq) on oviducts (Shu 2014). These data successfully identified several candidates for mucin core protein and glycan biosynthesis genes (Shu 2014), representing likely candidates for maternal effect genes. Future work aims to test whether these genes are under divergent selection along the acidification gradient.

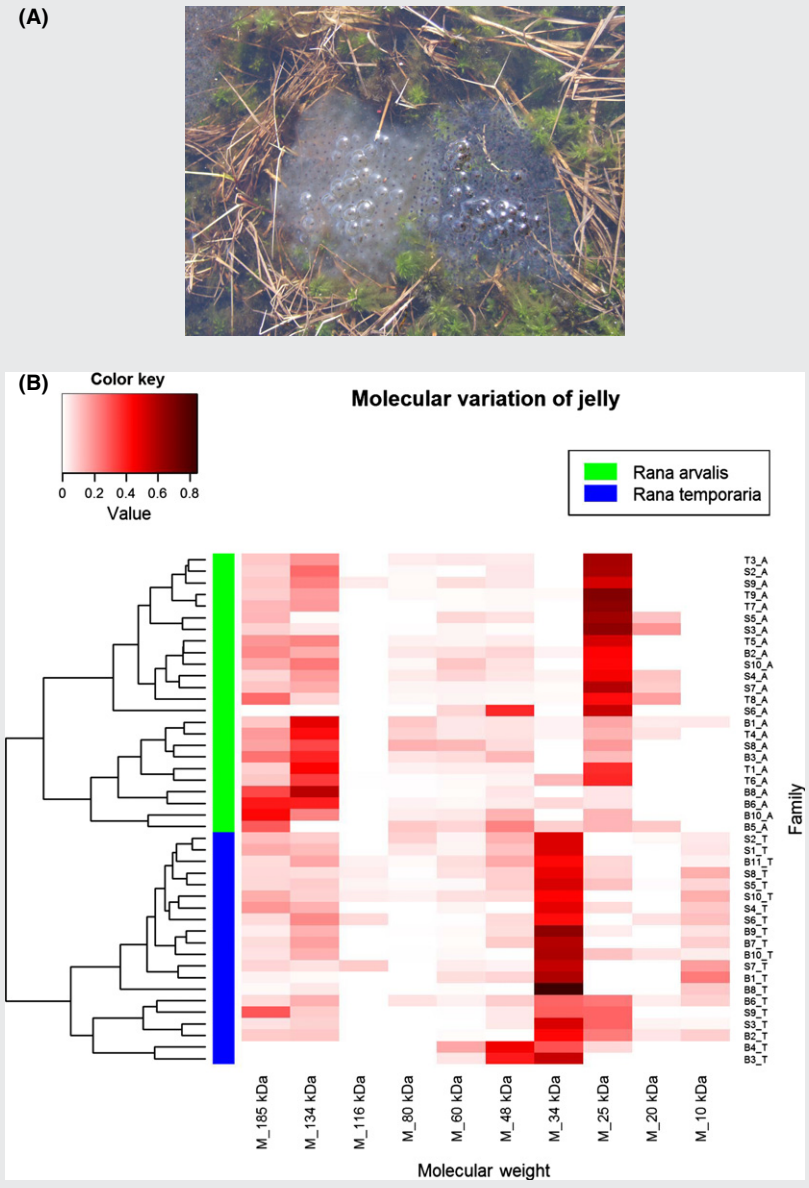
Although several questions are still open in our study system, the current evidence from RA (and RT) shows that jelly coats can harbour extensive intraspecific variation and strongly indicate jelly coat divergence in response to acidification. Key open questions that remain from this system are to what extent jelly coat variation within species are due to *genetically* based maternal effects, to what degree jelly coat variation reflects pH-mediated divergent selection among populations and what the molecular components of adaptive value are.

extent of phenotypic variation and the fitness consequences of this variation need to be understood as well.

Strategies and molecular tools for interdisciplinary studies

Modern molecular techniques allow identifying and quantifying different types of egg coat variations (Boxes 2 and 3). However, we are still often missing the link between the genes, the environment and the phenotype (Diz *et al.* 2012). This is important as variation in gene expression can directly cause variations in proteins and, subsequently, in the phenotype and function (Danchin *et al.* 2011). Second, extensive posttranslational modifications, such as glycosylation and phosphorylation (Carr 1997; Diz *et al.* 2012), are common in egg

Box 4. *Continued*



Box Fig. 4. (A) A clutch of *R. temporaria* (left) and *R. arvalis* (right), showing the species differences in jelly appearance. In aquatic breeding amphibians, such as RA and RT, jelly coats absorb an extensive amount of water when laid (e.g. Shu *et al.* 2015). At this swollen state, RA and RT differ somewhat in the appearance of the jelly coats, being much thicker and partially cloudy in RT and thinner and entirely clear in RA, but no obvious intraspecific variation is visible (Shu & Räsänen, pers. obs.). (B) Macromolecular variation of jelly coats from *R. arvalis* and *R. temporaria* based on SDS-PAGE analyses of composite jelly (i.e. the jelly has been isolated and solubilized for analysis, but the glycans and proteins have not been separated). The molecular masses of the gel bands are shown on the *x*-axis and the identity of the 48 clutches (i.e. family) on the *y*-axis (right). The colour scale indicates the expression of each band based on optical densities, which reflects the abundance of a given glycoprotein band. Clustering analyses highlighted two main clusters (left) reflecting the species (RA and RT). S, T and B indicate source populations that differ in breeding pond pH (S = neutral origin, T = acidic origin and B = intermediate pH population) (Shu 2014). As the SDS-PAGE gels here reflect the composite variation of glycoproteins, and most of this variation is due to glycans (rather than proteins), identification of variation necessitates the use of glycomics approaches (see Box 3 and 4 text).

coats. Posttranslational modifications cause alterations in the function of proteins coded by egg coat genes and can make it almost impossible to infer functional consequences based solely on DNA sequences (Danchin *et al.* 2011). This is particularly relevant for highly glycosylated glycoproteins (Bennett *et al.* 2012; Box 3).

Moreover, from an evolutionary ecological point of view, phenotypic plasticity often affects organismal responses to environmental heterogeneity (Ghalambor *et al.* 2007). In case of egg coats, such plastic effects probably arise as transgenerational plasticity via the mother (Agrawal *et al.* 1999; Herman & Sultan 2011). In model systems such as the domestic chicken (e.g. Hincke *et al.* 2012), the female environment has been shown to have strong effects on egg coat composition. However, to our knowledge, little is known about environment-dependent egg coat variability in natural populations. Techniques that allow quantifying molecular variation of egg coats at the phenotypic level and linking this variation to function and fitness (see next sections) are highly desired.

Measuring molecular variation of egg coats

Recent advances in mass spectrometry (MS) and molecular techniques, such as proteomics and glycomics (Lazar *et al.* 2011; Diz *et al.* 2012; Jensen *et al.* 2010, 2012), allow identifying egg coat proteins and glycans as well as quantifying their variation. For example, with a 'bottom-up' approach (from egg coat to amino acid sequence rather than amino acid sequence to egg coat), the proteome structures of oocyte coats have been identified in several vertebrates (e.g. chicken, Mann 2008; hamster, Izquierdo-Rico *et al.* 2009; rabbit, Stetson *et al.* 2012) and marine invertebrates (e.g. abalone, Aagaard *et al.* 2006, 2010, 2013; and ascidians, Yamada *et al.* 2009; echinoderms, Oulhen *et al.* 2013). Such quantification of protein variation, and identification of the underlying genes linked to this variation, is relatively well established and straightforward (Box 2). However, a large part of the molecular phenotype of egg coats – especially the jelly – consists of polysaccharides, which are much more difficult to analyse (Varki *et al.* 2009; Bennett *et al.* 2012; Joshi *et al.* 2015; Box 3).

Compared to the relatively well-studied oocyte coat proteins, the composition and structure of egg coat glycans largely remains unknown in most taxa (reviewed in Wong & Wessel 2006; Gallo & Constantini 2012), let alone the extent of intraspecific variation or the genetic basis of this variation. Fortunately, glycan profiles can now be analysed and compared with the aid of MS (Joshi *et al.* 2015; Box 3), as has been performed for jelly coats in several amphibian species (e.g. Guerardel *et al.* 2000; Delplace *et al.* 2002; Coppin *et al.* 2003; Li *et al.* 2011). High-resolution X-ray crystallography, which reveals the atomic architecture of proteins, allows further to characterize the three-dimensional structure, as well as predict the functions, of specific egg coat components (Monne *et al.* 2008; Han *et al.* 2010; Aagaard *et al.* 2013). A good overview of a combination of molecular approaches to understand the evolution of postzygotic egg coats is provided in Hincke *et al.* (2012) for avian egg shells. Finally, to make rigorous inferences about the evolutionary ecological processes influencing egg coat diversification, and their role in adaptation and speciation, a combination of molecular techniques, coupled with functional performance tests under ecologically relevant conditions, is needed (Turner & Hoekstra 2008a,b).

Evolutionary and functional analysis of egg coats

Phylogenetic and selection analyses on egg coat genes allow insight into the evolution of reproductive proteins (Findlay & Swanson 2009). To detect selection on egg coat genes (i.e. genes coding for egg coat glycoproteins and variations in them), the *dN*/*dS* ratio of the underlying DNA sequences (Hurst 2002) is often compared. Examples for this approach are evolutionary analyses of ZP genes in abalone (Box 2) and other taxa (e.g. humans, Männikkö *et al.* 2005; rodents, Turner & Hoekstra 2008b; sea urchins, Palumbi 2009; cetaceans, Amaral *et al.* 2011) and vitelline membrane and chorion genes of *Drosophila* (Jagadeeshan & Singh 2007). However, as DNA sequence-based tests may reveal little about the functional effects of evolutionary changes in egg coats, such genetic analyses of selection should be integrated with the functional analysis. Inferring glycan-mediated selection on egg coats (particularly jelly coats, which typically consist largely of mucin-type O-linked glycans) certainly will require a multidisciplinary approach (Bennett *et al.* 2012; Joshi *et al.* 2015; Nairn & Moremen 2015).

Functional analyses of egg coats have mostly applied various biochemical approaches and sperm-binding assays to identify the role of egg coats during fertilization (e.g. Segall & Lennarz 1979; Bonnell *et al.* 1996). In model systems, various genetic engineering techniques provide powerful tools to establish the functional role of observed molecular variations in egg coats. Gene knockin and knockout experiments, and the use of transgenic lines, have helped to elucidate the zona pellucida structure and function in the house mouse (*M. musculus*) (e.g. Rankin *et al.* 1996, 2003; Gahlay *et al.* 2010). Cutting-edge genome editing techniques, such as zinc-finger nucleases (ZFNs) (Carroll 2011), transcription activator-like effector nucleases (TALENs) (Joung & Sander 2013) and clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based system (Sander

& Joung 2014), are promising avenues for genetic manipulations in model systems (Gaj *et al.* 2013).

In natural populations amenable for experiments, it should be possible to establish fitness consequences of egg coat variations in different ecologically relevant contexts, and when combined with molecular techniques, establish the links among molecular variation, function and fitness within and between species. For instance, quantitative genetic crosses, combined with egg coat manipulation experiments, within and between divergent populations can help to establish the relative contribution of maternal and direct genetic effects on offspring performance and the role of the composite egg coats for embryonic fitness (Box 4). As egg coat-mediated effects may strongly depend on environmental conditions, experiments should include rearing of embryos from different populations under a range of ecological conditions (e.g. Hangartner *et al.* 2012) and testing for the performance of different egg coat variants in the wild (e.g. Linnen *et al.* 2013). In taxa where cross-generational rearing is possible, studies over multiple generations and experimental evolution approaches may be particularly useful in investigating the evolutionary consequences of transgenerational plasticity (e.g. Zhou *et al.* 2012), such as are likely in maternally derived egg coats. When different genotypes (e.g. individuals from divergent populations) are compared over generations, it is possible to investigate genetic variation in transgenerational plasticity (e.g. Herman & Sultan 2011) – and when combined with artificial selection, evolutionary consequences of different egg coat variants could be tested. Finally, tissue-specific transcriptomics (i.e. on sites of egg coat production; Box 1) can allow identifying genes expressed during egg coat production and, when combined with exposing females to different ecological conditions (Box 2), infer variation in egg coat-related gene expression. Subsequently, the consequences of the maternal environment on egg coat composition and offspring performance could be experimentally tested. Ultimately, a combination of molecular tools (Boxes 2 and 3) and functional assays (Boxes 2, 3 and 4) should make it possible to infer how egg coat evolution is influenced by both sperm–egg interactions and natural selection acting via embryonic performance also in natural populations.

Concluding remarks

In this review, we summarized the current progress on egg coat studies and identified research gaps and challenges. We particularly emphasize the need for incorporating studies on natural selection via embryonic performance and intraspecific variation. Given maternal transmission and importance of molecular-level variations in egg coats, interdisciplinary studies that link different molecular and evolutionary ecological experimental approaches are needed to quantify intraspecific variation in egg coats and the consequences of this variation for organismal fitness. In doing so, we can make substantial progress in understanding the underlying factors behind the immense variability in structural and molecular composition of egg coats across and within taxa, and their role in the evolution of biological diversity – in particular in relation to maternal effects, adaptation and speciation.

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Glossary

Acrosome reaction: the process by which the contents of the sperm acrosome are released, facilitating the fusion of the sperm with the egg plasma membrane.

***De novo* sequencing**: peptide sequencing performed without prior knowledge of the amino acid sequence. Often performed in nonmodel species.

dN/dS ratio: the ratio of nonsynonymous coding sequence substitutions at nonsynonymous sites (dN) to synonymous (silent) coding sequence substitutions at synonymous sites (dS). A ratio >1 indicates positive selection, <1 indicates purifying selection, and a ratio of 1 indicates neutral substitution.

Egg coat: the complete extracellular structure surrounding the embryo. These structures are produced by the mother either before or after fertilization (see Box 1).

Fertilization envelope (FE): a glycoprotein membrane surrounding the plasma membrane of a zygote.

Fertilization: the fusion of gametes to produce a new organism. This process can be either internal (within the body of the female) or external (outside the body of the female). Egg coats play a fundamental role in sperm–egg interactions.

Genome editing: a genetic engineering technique. Normally, DNA is inserted, replaced or removed from a genome using artificial engineering.

Glycome: the entire set of oligosaccharides of a given cell, tissue or organism.

Glycomics: the comprehensive study of the entire glycan (oligosaccharide) structure of a given glycome.

Glycoprotein: a protein that contains covalently attached oligosaccharide chains.

Glycosylation: an enzymatic process that attaches glycans to proteins, lipids or other organic molecules.

Jelly coat: a thick sticky gelatinous structure surrounding the oocyte coat in some taxa. Jelly coats are highly glycosylated (i.e. consist primarily of oligosaccharides).

Knockin and knockout techniques: genetic engineering methods that allow inserting a protein-coding cDNA sequence into a particular locus of a species (knockin) or making a given gene inoperative (knockout).

Mass spectrometry (MS): an analytical technique that measures the mass-to-charge ratio of charged molecules and their fragments (MS/MS). It is widely used to identify chemical structures and peptide sequences and to quantify organic molecules.

Maternal effects: the effect of a mother's phenotype and environment on offspring phenotype and performance. Maternal effects can either be environmentally induced or have a genetic basis.

Oocyte: the immature germ line cell of the mother (prior to fertilization).

Perivitelline space: the space between the oocyte coat and the embryo. This space may be filled with a fluid, have functional consequence in sperm–egg interactions and mediate interactions between the embryo and the egg coats.

Proteome: the entire set of proteins of a given cell type, tissue or organism.

Proteomics: The large-scale study of proteins, including their structure, function and interactions.

Pseudogenes: dysfunctional genes that have lost their protein-coding ability during evolution or are no longer expressed.

Posttranslational modification (PTM): the chemical modification (e.g. glycosylation, phosphorylation) of a protein after its translation. Glycoproteins are typical examples of a PTM.

RNA-seq: a technology that uses next-generation sequencing to detect RNA presence and quantity from a genome.

SDS-PAGE: sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

Transcriptome: the full set of messenger RNA (mRNA) molecules expressed by an organism or tissue.

Vitelline envelope (VE): a glycoprotein membrane surrounding the plasma membrane of an unfertilized egg.

X-ray crystallography: a method of determining the atomic and molecular structure of a crystal. This method can aid structural and functional analyses of proteins.

Zeta potential: the electric potential across the double layer of a charged particle or molecule in solution.

Zona pellucida (ZP) domain: a family of evolutionarily related proteins. ZP glycoproteins share a common structural motif, known as the ZP domain.

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K.R. had the initial idea for the manuscript. L.S. wrote the first draft of the manuscript, prepared figures and tables and extracted gene data from GenBank. M.J.F.S. provided expertises on molecular analyses. All authors contributed to the writing of the manuscript.

Data accessibility

Data on ZP genes used in the manuscript was retrieved from GenBank. GenBank Accession nos are provided in Table S1 (Supporting information).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 List of ZP genes used in Fig. 1.