Review

Genetic adaptations for the oceanic success of fish eggs

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Genetic adaptations of organisms living in extreme environments are fundamental to our understanding of where life can evolve. Water is the single limiting parameter in this regard, yet when released in the oceans, the single-celled eggs of marine bony fishes (teleosts) have no means of acquiring it. They are strongly hyposmotic to seawater and lack osmoregulatory systems. Paradoxically, modern teleosts successfully release vast quantities of eggs in the extreme saline environment and recorded the most explosive radiation in vertebrate history. Here, we highlight key genetic adaptations that evolved to solve this paradox by filling the pre-ovulated eggs with water. The degree of water acquisition is uniquely prevalent to marine teleosts, permitting the survival and oceanic dispersal of their eggs.

Highlights

Since recolonizing the seas, most teleosts evolved as pelagophils, producing highly hydrated pelagic (buoyant) eggs.

Osmotic drive for oocyte hydration is mainly generated through differential proteolysis of vitellogenin-Aa-derived yolk proteins.

Teleost-specific water channels are selectively retained in pelagophils and coevolved with 14-3-3ζ-like binding proteins to co-operatively regulate channel trafficking in a manner that avoids competitive membrane space occupancy and accelerates oocyte water influx.

Gonadotropinergic and vasopressinergic systemic and paracrine signaling systems converge to activate meiosis resumption and oocyte hydration.

Developmental downregulation of dominant-negative inhibitory aquaporin variants maximizes oocyte hydration.

These innovative genetic adaptations are highly conserved in modern marine teleosts, contributing to the success of their eggs in the oceans.

Adaptive radiation in the oceanic environment

The true spiny ray-finned fishes (Euacanthomorphacea) are the most diverse group of vertebrates, comprising almost a third of all species (Box 1). The fossil record revealed that their explosive radiation occurred during the early **Paleogene** (see Glossary), predominantly in marine environments, resulting in a historical diversification rate that eclipsed the evolution of birds and mammals [1–3]. The success of the euacanthomorph teleosts in the oceans is, however, surprising since their ancestors are considered to have evolved in freshwater [4,5]. As for all teleosts, their body fluids are strongly hyposmotic to the desiccating environment of seawater, which is a rare condition amongst animal taxa and is argued to be a physiological vestige of their freshwater ancestry [6,7].

To truly conquer the oceans, it is inevitably necessary to reproduce there. This posed a major physiological problem for the single-celled eggs of teleosts, which are equally hyposmotic to seawater when released at spawning [8]. In contrast to the juveniles and adults, which drink seawater and regulate excess salt loads via the intestine, kidney, and gills [9], the single-celled eggs are devoid of such **osmoregulatory organ** systems and thus incapable of acquiring the essential parameter for life: water. As noted by Fyhn and colleagues [10], teleosts pre-adapted the embryos by adding water to the oocyte during the maturational phase of **meiosis** resumption to resolve this. The process is known as oocyte hydration, with the water subsequently remaining locked within the egg due to the reduced permeability of the **vitelline membrane** until osmoregulatory systems develop in the embryo. The degree of hydration varies depending upon whether the eggs float freely in the oceanic currents (pelagic eggs), sink to the sea floor or become attached to substrates (benthic eggs), or are incubated internally by the parent. The latter eggs do not undergo substantial hydration, as they are afforded protection from seawater within a variety of parental organs that are maintained close to isosmolality with the plasma [11–13]. Conversely, benthic eggs that are released in seawater by benthophils typically hydrate up to ~80% of wet mass, while pelagic eggs, produced by pelagophils, acquire water contents of ~90% or more, reducing their specific gravity below that of the saline environment and rendering them buoyant [14].

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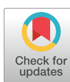

Box 1. Actinopterygian (ray-finned fish) evolution

Actinopterygian fishes originated in the late Silurian after separating from the lobe-finned lineage (Sarcopterygii) that gave rise to tetrapods [77]. Ancestral actinopterygians from which the extant bichirs (Cladistia) are descended further diverged in the Devonian Period (Figure I). Subsequent diversifications in the Carboniferous Period gave rise to the ancestors of sturgeons and paddlefishes (Chondrostei) as well as the first new-finned (Neopterygii) species from which extant gars and bowfin (Holostei) and teleosts (Teleostei) evolved [77]. In terms of diversity, however, the pre-teleost lineages only account for 0.1% of modern actinopterygians, while teleosts account for the rest (99.9%). Teleosts thus represent 96% of all fishes and approximately half of all vertebrates. Prior to the diversification of teleosts, their last common ancestor underwent a whole genome duplication (WGD) event (R3) [78], which doubled the gene repertoires in relation to the pre-teleost lineages. During subsequent evolution, however, a process of delayed rediploidization differentially reduced R3-generated gene copy numbers by ~70–80% [79]. Extant teleosts diverged in four cohorts: Elopomorpha, Osteoglossomorpha, Otomorpha, and Euteleosteomorpha; however, the majority of teleost diversity is not evenly distributed, it is dominated by two clades: the freshwater Ostariophysi and the predominantly marine Euacanthomorphacea. The latter clade explosively diversified during the early Paleogene ~245 million years after the R3 WGD with modern family-level diversity established within the first 10 million years following the Chicxulub impact event [1–3].

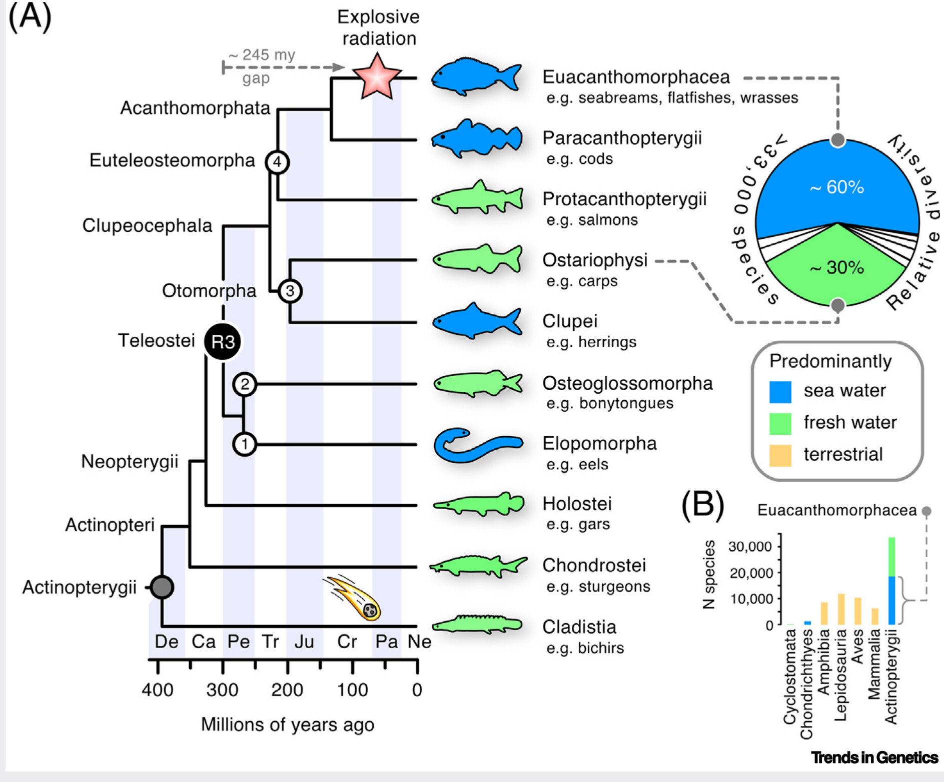

Figure I. Actinopterygian (ray-finned fish) evolution. (A) Simplified overview of the evolutionary relationships of actinopterygian fishes (see [80,81] for more complete overviews). Diversification times are median values from timetree.org. (B) Modern Euacanthomorphacea represent almost a third of all vertebrates. Statistics are from https://www.mammaldiversity.org/, https://avibase.bsc-eoc.org/, https://reptile-database.org/, https://amphibiaweb.org/, and https://fishbase.se/. Abbreviations: Ca, Carboniferous; Cr, Cretaceous; De, Devonian; Ju, Jurassic; Ne, Neogene; Pa, Paleogene; Pe, Permian; Tr, Triassic.

The reproductive trait of producing pelagic eggs is not recorded in the fossil record but has clearly been selectively advantageous for the success of euacanthomorph marine teleosts. The implications were recognized more than a century ago by Fulton following the discovery of oocyte hydration 'which has, so to speak, in the pelagic egg, been seized upon and exaggerated by natural selection for another purpose – namely to enable eggs to float and become widely dispersed' [15]. This

Glossary

Amniota: a subgroup of tetrapods (reptiles, birds, mammals) that form a fluid-filled extra-embryonic sac (the amnion) during embryogenesis.
Dominant-negative inhibition: a process in which the function of a wild-type gene is preferentially inhibited by the coexpression of its variant in a dose response-dependent manner.
Endocytosis: a process in which extracellular cargo is internalized at the cell surface with the formation of membrane-bound vesicles.
Endosome: intracellular membrane-bound organelles involved with the sorting and delivery of cargo.
Exaptation: a mechanism or trait that evolved for one purpose but is co-opted to perform an accessory or different function.
Isoform: variant of a single gene and thus differing in relation to the normal (canonical) gene.
Meiosis: a process in which two rounds of cell division halve the number of chromosomes to produce haploid gametes. During prophase of the first round, duplicated homologous chromosomes align and may exchange genetic information resulting in a recombined genome.
Metazoa: multicellular animals with differentiated tissues.
Microlecithal: microlecithal, mesolecithal, and megalecithal, respectively, refer to the small (e.g., non-eutherian mammals), medium (e.g., amphibians), or large (e.g., fishes, reptiles, birds) amounts of yolk deposited in their eggs.
Ortholog: genes that have a common origin in different organisms.
Osmoregulatory organ: specialized cells and organs that actively regulate body fluid water and salt content.
p38 MAPK: a class of mitogen-activated protein kinase involved in protein phosphorylation and signal transduction cascades.
Paleogene: the earliest period (geological time subdivision) of the Cenozoic Era. It began after the Cretaceous Period ended 66 million years ago and includes the Paleocene, Eocene, and Oligocene Epochs.
Paralog: the set of related genes within an organism.
Polyuria: excessive urination volume.
14-3-3 Proteins: eukaryotic family of activation molecules that can bind to diverse proteins to illicit different cellular

exaptation has indeed been seized upon by modern euacanthomorph teleosts, the majority of which, regardless of their systematic affinities, coastal or oceanic distribution, tropical or boreal ranges, or demersal or pelagic habitats, spawn pelagic eggs [16,17]. Moreover, pelagic eggs are typically released in multiple batches in vast quantities (up to ~300 million developing in the ovaries of some species [18]), with each zygotic individual housing two recombined genomes. It is with such novel genetic variety and adaptive potential that the legions of eggs are geodispersed as passive passengers in the oceanic currents. Historically, pelagophils may have evolved as long ago as the **Triassic** in the ancestors of elopomorph teleosts, but in that period they swam in the shadow of great predators such as *Saurichthys*, *Birgeria*, and others of reptilian descent [19]. By contrast, modern family level diversity of the Euacanthomorphacea was established within the first 10 million years after the Cretaceous–Paleogene extinction event, which decimated the marine predators [1–3]. The sudden reduction in predation pressure may therefore have provided the opportunistic ecospace for the flotillas of teleost eggs in the oceans [20,21].

Physiological mechanisms for oocyte hydration

The primary driving force for the uptake of water in marine teleost eggs is caused by changes in the intra-oocytic osmotic pressure resulting from increased levels of inorganic ions and free amino acids (FAA) occurring during meiosis resumption (Box 2). In both benthic and pelagic eggs, the major inorganic osmotic effectors are chloride (Cl^-), potassium (K^+), phosphate (P_i), and ammonium ions (NH_4^+) [8,14,22]. However, although Na^+,K^+-ATPases are implicated [14], little is known of the molecular mechanisms regulating such inorganic ion fluxes. By contrast, the genetic origin of the organic osmolyte pool of FAA has been well documented. It is derived from the differential proteolysis of selected yolk proteins. To provide insight into how such an organic osmolyte pool arose, we briefly review yolk protein deposition and subsequently discuss the main genetic adaptations that selectively evolved to generate high concentrations of FAA in pelagic eggs.

Box 2. Endocrine regulation of egg formation in teleosts

In oviparous vertebrates, such as fish and amphibians, oogonia enter into meiosis (until prophase of the first meiotic division) to become primary oocytes and subsequently form a follicle when they are surrounded by somatic cells, which will later differentiate into theca and granulosa cells. Further growth of the oocyte occurs mainly by the uptake of large quantities of vitellogenins (Vtgs), lipoproteins, and vitamins from the bloodstream, which are accumulated in the oocyte cytoplasm. After the growth phase, oocytes complete the first meiotic division followed by progression to metaphase of the second meiotic division, a process commonly termed ‘oocyte maturation’, and are subsequently released from the enveloping layer of somatic cells (ovulation), resulting in the production of a fertilizable egg.

Ovarian development in teleosts is mainly regulated by the pituitary gonadotropins follicle-stimulating and luteinizing hormones (Fsh and Lh, respectively), which act on the ovary through their cognate receptors, the Fsh receptor (Fshr) and the Lh/choriogonadotropin receptor (Lhcgr) [82] (Figure I). In general, the major regulator of the oocyte growth phase is Fsh, which induces the synthesis of estradiol-17β (E2) in the follicular cells surrounding the oocyte through Fshr activation. E2 secretion into the bloodstream activates the hepatic synthesis and release of the Vtgs, which are internalized by growing oocytes [25]. Ovarian follicle production of E2 can also maintain the meiotic arrest of oocytes through the activation of a G protein-coupled estrogen receptor on the oocyte surface, resulting in cAMP production and the maintenance of elevated cAMP levels for protein kinase A (PKA) activation [83]. In fully grown oocytes, an adenohypophysial surge of Lh activates increased levels of Lhcgr in ovarian follicles to induce the steroidogenic shift from E2 to C_{21} steroids (progestins) production in granulosa cells [84]. The progestins 17α, 20β-dihydroxy-4-pregnen-3-one (17,20β-P) or 17α, 20β, 21-trihydroxy-4-pregnen-3-one (20βS) are typically the maturation-inducing hormones in fish, which bind a G protein-coupled membrane receptor on the oocyte surface (mPgr α and/or β), triggering the activation of the maturation promoting factor (MPF) and the resumption of meiosis [84,85]. In addition to nuclear events related to meiosis resumption, the process of oocyte maturation is accompanied by several changes in the oocyte cytoplasm, such as lipid coalescence, the partial or complete fusion of yolk globules, together with the disassembly of their crystalline-like structure and, in marine pelagophil teleosts, a massive water uptake (hydration).

Finally, beyond the progestin control of oocyte maturation, other hormones, including growth factors, neuropeptides, and neurotransmitters, can also play a role in this process [86–88]. These factors can either regulate the steroidogenic function of follicular cells mediated by Lh, or the action of progestins in the oocyte, in a systemic or paracrine manner.

processes, including the regulation of the intracellular trafficking of membrane proteins.

Triassic: the first period of the Mesozoic Era.

Vitelline membrane: the plasma membrane of a developing oocyte/egg. It is sometimes called the plasmalemma or oolemma. It is distinct from the vitelline envelope (also termed chorion, zona pellucida), which is typically comprised of zona proteins that form an acellular outer surrounding to the egg.

Vitellogenin (Vtg): the origin (genesis) of yolk (vitellus) proteins. Five types of major yolk proteins evolved in animals, each associated with the processing or cellular internalization of cargo: the lipid-bearing vitellogenins of metazoans, including many protostomes and vertebrates; the transferrin-derived yolk proteins of echinoderms (e.g., sea urchins) and cephalochordates (lancelets); the apolipophorin-derived yolk proteins of crustaceans; and the two lipase-derivatives of butterflies (Lepidoptera) and fruit flies (Diptera).

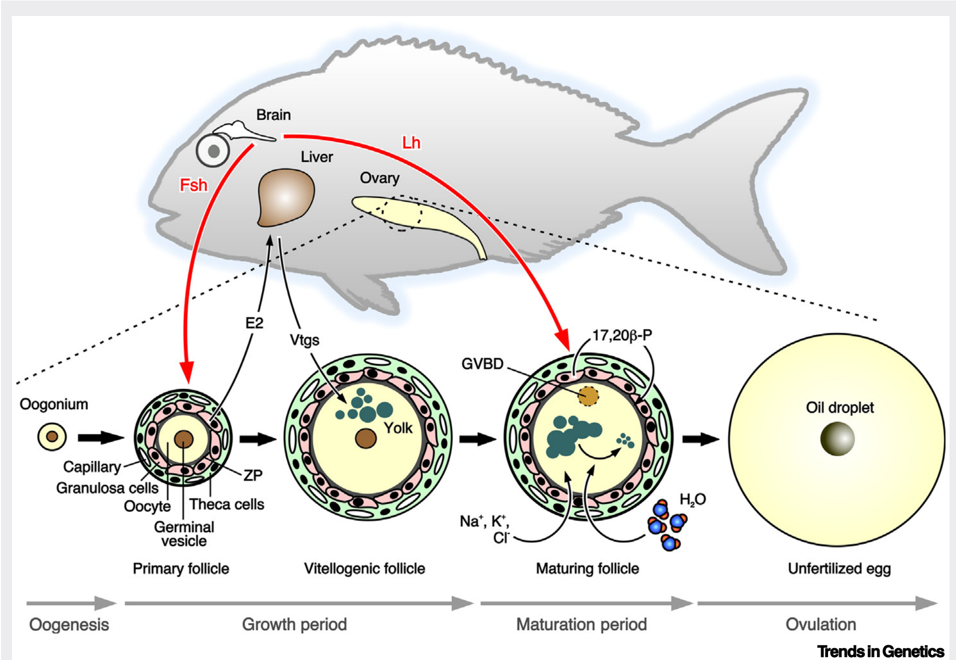

Figure I. Endocrine regulation of oocyte growth and maturation in teleosts. Oogonia enter into meiosis until prophase I to become primary oocytes, which associate with follicle theca and granulosa cells. Follicle cells surrounding the oocytes are stimulated by follicle-stimulating hormone (Fsh) to produce and release estradiol-17β (E2), which induces hepatic vitellogenin (Vtg) synthesis. The Vtgs are endocytosed into the oocyte and processed into yolk proteins for storage. When vitellogenesis resumes, maturation-inducing progestins synthesized by follicle cells in response to luteinizing hormone (Lh) activate the membrane progestin receptor (mPgr) on the oocyte surface, triggering meiosis resumption. This process coincides with the proteolysis of selected yolk proteins, the accumulation of inorganic ions, and the hydration of the oocyte. Abbreviations: GVBD, germinal vesicle breakdown; ZP, zona pellucida.

Evolution of yolk precursors

The major yolk proteins of vertebrates are derived from **vitellogenin (Vtg)** precursors encoded by a set of genes that have been conserved since the origin of **Metazoa** (see later). Gene translation of vertebrate **orthologs** produces multidomain glycophospho-apolipoproteins that fold to form a flexible lipid-binding cavity comprised of lipovitellin heavy (LvH) and light (LvL) chains [23]. In primary structure, the LvH and LvL domains are separated by a polyserine-rich phosvitin (Pv) region of lineage- and **paralog**-specific length and degree of phosphorylation that forms a hydrophilic sail around the molecule, followed by a C-terminal domain homologous to the von Willebrand factor type D (Vwfd) (Figure 1A). In teleosts, the Vwfd may be cleaved into a beta component (β′) and a C-terminal coding segment (CT), with some derived Vtgs secondarily losing the Pv, β′, and/or the CT subdomains [24].

Extra-ovarian Vtg synthesis occurs in the liver in response to estrogen (17β-estradiol) secreted by the follicular cells that are in turn activated by the adenohypophysial gonadotropin follicle-stimulating hormone (Fsh) (Box 2). Subsequent to systemic circulation as a dimer, Vtg binds to specific receptors (Vtgr) on the oocyte surface and is internalized via clathrin-mediated **endocytosis** to initially form membrane-bound coated vesicles [25,26]. With the removal of the clathrin coat, the vesicles coalesce in early **endosome**s to form yolk globules, which in many vertebrates, including teleosts, can subsequently mature into yolk platelets carrying pseudocrystal lattices (see Outstanding questions) [27–29]. During this stage, termed the vitellogenic period, proton pumps (H^+-ATPase) mildly acidify the endosomal lumen resulting in the cleavage of Vtg at conserved sites

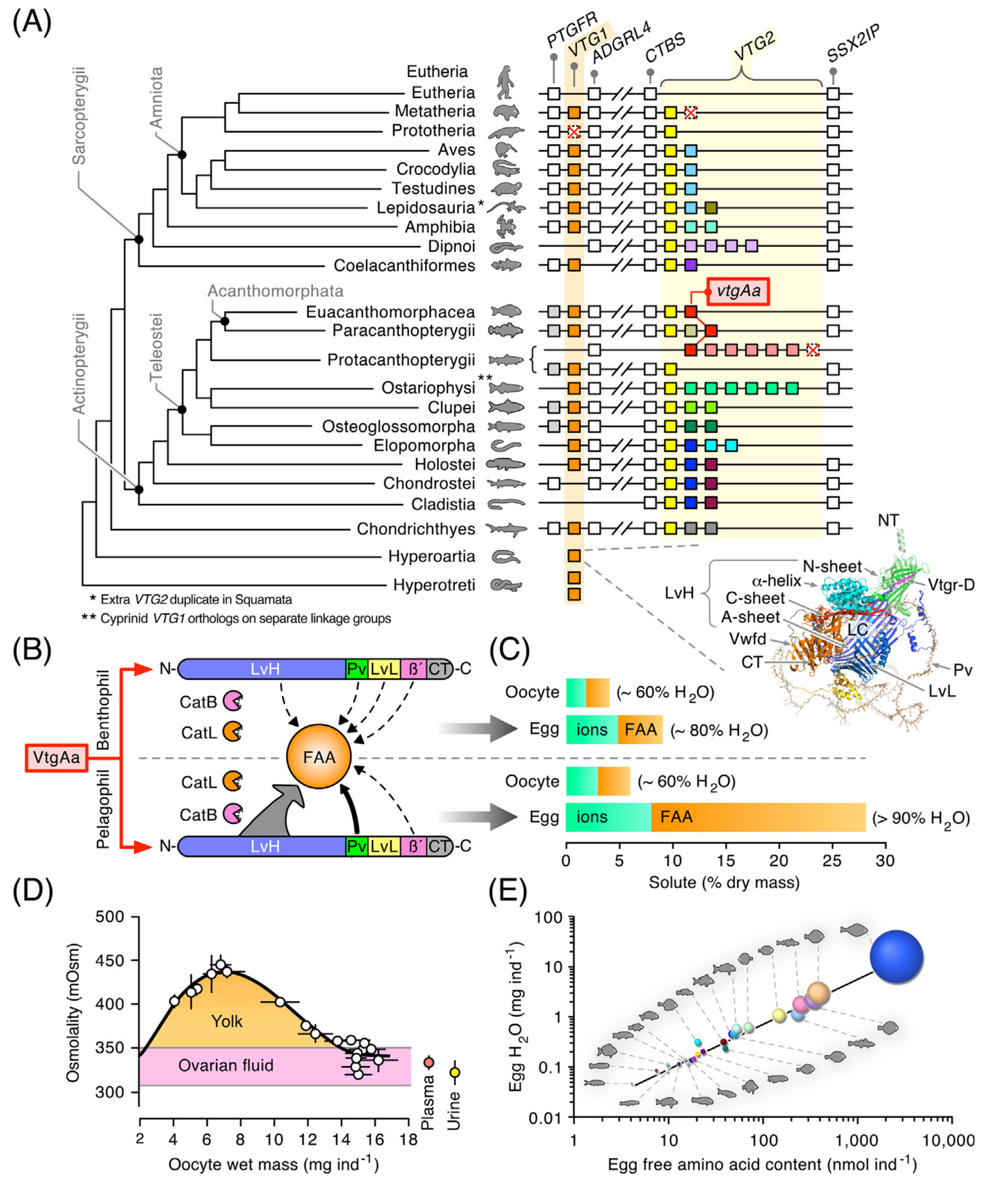

Figure 1. Yolk proteins evolved to generate intra-oocytic osmotic pressure. (A) Syntenic arrangement of vertebrate vitellogenin (*VTG*) genes (not to scale). Linkage groups are oriented in accordance with the flanking orthologs (white squares) *PTGFR*, *ADGRL4*, *CTBS*, and *SSX2IP* on human Chr1. Gray squares in Teleostei indicate *ptger*. Orthologous *VTG* gene squares (same color) are inferred from the Bayesian phylogenetic tree (Figure S1 in the supplemental information online). A *VTG2*-type duplicate (*vtgAa*) evolved in Euteleostei and neofunctionalized in Acanthomorphata (see Box 1 in the main text) to produce yolk proteins that are differentially proteolyzed to free amino acids (FAA) for the generation of intra-oocytic osmotic pressure. The monomeric model of lamprey Vtg (UniProt: Q91062) is downloaded from Alpha-Fold (https://alphafold.ebi.ac.uk/) and rendered in PyMOL (https://pymol.org/). The amino- (NT) and carboxy- (CT) termini, lipovitellin heavy (LvH) and light (LvL) chains, phosvitin (Pv), and von Willebrand factor type D domain (Vwfd) are indicated. The lipid cavity (LC) is shown in the center of the molecule and the Vtg receptor domain (Vtgr-D) is located in the N-sheet. (B) Cathepsins (CatB and CatL) differentially proteolyze the LvH and other yolk proteins derived from VtgAa more extensively in pelagophils compared with benthophils (modified from [62]). (C) Resultant FAA contents in pelagic eggs far exceed those of benthic eggs, with inorganic ions (Cl^-, K^+, P_i, and NH_4^+) also contributing osmotic drive for the hydration mechanism (modified from [22]). (D) Transient hyperosmolality of the yolk in relation to the ovarian fluid, plasma, and urine during oocyte maturation of a marine pelagophil (modified from [8]). (E) The amount of egg water of euacanthomorph marine pelagophils is allometrically related to the FAA content ($y = 0.0106x^{0.904}$; $r^2 = 0.98$) (modified from [44]). Data points are scaled in accordance with egg wet mass.

into the constituent yolk proteins, by the action of lysosomal hydrolases such as cathepsins [24,30–32]. Yolk proteins are subsequently stored until embryogenesis (see Outstanding questions), when a secondary acidification reactivates the lysosomal hydrolases to commence yolk proteolysis [32–34]. Specifically, within the eggs of marine teleosts, however, and particularly acanthomorph pelagophils, this secondary phase of yolk processing is advanced to the pre-ovulatory stage of meiosis resumption to drive oocyte hydration, but predominantly through the proteolysis of specific yolk proteins. To understand which yolk proteins are degraded we briefly review the genetic adaptations associated with Vtg evolution.

Unique forms of Vtg evolved in vertebrates

The earliest form of Vtg can currently be traced to the last common ancestor of Metazoa, with orthologs detected in sponges (GenBank accession no. MW685966), corals, and jellyfishes [35]. In jawless vertebrates (Agnatha) two genes exist in hagfishes (Hyperotreti), but only a single ortholog is found in lampreys (Hyperoartia). Conversely, in jawed vertebrates (Gnathostomata), two sets of *vtg* genes (*vtg1* and *vtg2*) are located in closely linked regions of the genomes [36,37] (Figure 1A). Previous phylogenetic analyses revealed that the *vtg1* genes (also termed *vtgC* or *vtg3* in actinopterygians) and at least one of the *vtg2* genes are orthologous [38–41]. However, the *vtg2* genes independently duplicated to generate orthologous forms in the **Amniota** and basal non-teleost actinopterygians, respectively, with one of the latter orthologs (*vtg2a*) identified in elopomorph tarpons, but seemingly lost in the other teleost cohorts (Figure S1 in the supplemental information online). In metatherian (marsupial) and eutherian (placental) mammals, the *VTG1* and *VTG2* genes were reportedly inactivated with the evolution of lactation and placentation [42]. However, a review of the molecular phylogenetic relationships and synteny of the vertebrate *vtgs* shows that some australidelphian metatherians (the common brushtail possum, *Trichosurus vulpecula*, and monito del monte, *Dromiciops gliroides*) in fact transcribe the *VTG1* and *VTG2* orthologs, with the deduced proteins clustering in the expected topological positions (Figure S1). This suggests that the *VTG* genes of some metatherians may have remained active to furnish the **microlecithal** eggs with yolk. In anamniotic vertebrates, additional *vtg2*-type duplications further generated nonorthologous forms in chondrichthyans, coelacanths, lungfishes, amphibians, squamates, basal actinopterygians, and teleosts. Amongst these novel forms in teleosts is the *vtgAa* gene that was first considered specific to the Acanthomorphata [20]. However, the revision of the molecular phylogeny of vertebrate Vtgs (Figure S1), shows that the *vtgAa* gene has a common ancestry in the Euteleosteomorpha (Figure 1A). This includes the protacanthopterygian salmonids, which enter seawater as smoltified juveniles, but do not spawn there, since their eggs are incapable of surviving in the hyperosmotic environment [43]. As in the pre-teleost freshwater actinopterygian lineages, salmonids do not degrade their yolk proteins during meiosis resumption and their oocytes do not undergo substantial oocyte hydration [44] and, consequently, they are bound by their eggs to return to their nascent rivers to maintain an anadromous lifestyle [20]. This is not the case for the highly diverse marine Acanthomorphata, which did evolve solutions for reproducing in the oceans. It is primarily the yolk proteins derived from the LvH of VtgAa that are differentially proteolyzed to generate the organic osmolyte pool of FAA that drives oocyte hydration (Figure 1B–E).

Mechanisms of differential proteolysis of yolk proteins during oocyte hydration

At the culmination of vitellogenesis, a suite of intracellular transduction cascades is activated in response to a range of signaling molecules, including the pituital secretion of the luteinizing hormone (Lh) and nonapeptides such as arginine vasopressin (Avp, formerly termed vasotocin) (Box 2). The resulting synthesis of maturation-inducing progestins triggers germinal vesicle breakdown (GVBD), meiosis resumption, and the events that generate the osmotic drive and water flow into the hydrating oocyte. Amongst these events is the entry of inorganic ions, the reactivation of

H^+-ATPases, acid phosphatases, and cysteine proteases (e.g., cathepsins B and L) in the yolk globules, and their fusion and dissolution [32,45–47]. The entry of K^+ is thought to destabilize the pseudocrystalline lattice when present, while the inorganic phosphate that arises likely stems from the dephosphorylation of Pv components [45,48]. In pelagophils, the increased hydrolytic enzymic activity liberates FAA primarily from the LvH, but also from the Pv and β′ subdomains of the VtgAa-derived yolk proteins and, to a much lesser extent, from the VtgAb derivatives [49–52] (Figure 1B). This leaves the VtgAb-derived proteins mostly intact for subsequent embryonic nutrition. Conversely, in benthophils only limited or no maturational proteolysis occurs [46,53–55]. As a result, a much larger pool of FAA is generated in the eggs of pelagophils (Figure 1C), with the time-course of proteolysis driving a temporal hyperosmolality of the yolk in relation to the ovarian fluid (Figure 1D). The mechanism is highly conserved in acanthomorph marine pelagophils, with the amount of water acquired highly significantly (r^2 = 0.98) related to the egg FAA content across four orders of magnitude (Figure 1E). Consequently, the main genetic adaptations that evolved to generate the osmotic drive in marine pelagophil oocytes are those associated with the evolution and extensive proteolysis of the VtgAa-derived yolk proteins. In some species that produce smaller eggs, this adaptation may have been selectively enhanced, with VtgAa almost exclusively deposited in the oocytes [56]. Nevertheless, the molecular basis for the differential degradation of yolk proteins cleaved from VtgAa and not VtgAb remain largely unknown (see Outstanding questions). Current hypotheses suggest their differential compartmentalization during receptor-mediated uptake as the underlying mechanism [25,57], but this remains to be demonstrated.

Aquaporins mediate oocyte hydration in pelagophil teleosts

Although water can passively traverse biological membranes via simple diffusion when exposed to an osmotic gradient, it has been known since the 18th century that the flux rate is substantially elevated in certain tissues, such as the ventral skin of amphibians, when exposed to neurohypophysial peptides [58]. Similar observations in mammalian red blood cells eventually led to the discovery of aquaporin-1 (AQP1), which elicits an approximately eightfold increase in transmembrane water flux when expressed in *Xenopus laevis* oocytes [59]. Water flow into the hydrating oocytes of marine pelagophils far exceeds the rate of diffusion (greater than sevenfold) implying that a facilitated mechanism for its transmembrane flux should exist. Such a pathway was first discovered in the oocytes of the gilthead seabream (*Sparus aurata*) in which a teleost-specific aquaporin (Aqp1ab, originally termed Aqp1o) related to mammalian AQP1 (Box 3) facilitates hydration within ~2 h [60,61]. Related Aqp1ab channels have subsequently been identified in a range of pelagophils and are thus considered to be the main conduits for water influx to the hydrating oocytes [62]. However, very recently it was discovered that a second Aqp1ab-type channel that evolved via tandem duplication >300 million years ago (MA) within a teleost-specific aquaporin-1 cluster (TSA1C) also facilitates the process [63] (see Figure IB in Box 3). These channels are termed Aqp1ab1 and Aqp1ab2 in accordance with the chronology of their discovery. The tandem evolution of two such closely related water channels that perform the same function in the same plasma membrane is unusual. For example, although clustered water channel genes such as *AQP2*, *-5*, and *-6* [64,65] are each expressed in the collecting ducts of mammalian kidneys, or amphibian ventral pelvic skin patches, and *AQP3* and *-7* are coexpressed in the apical membranes of the epithelial cell microvilli of the large intestine, they are more typically localized in separate cells or compartments and are not noted to compete for the same membrane space [66–68]. Indeed, when such colocalization arises in humans with **polyuria**, it has been shown that AQP5 interacts with AQP2 and impairs its cell surface localization [69]. In teleosts, however, a remarkable feature of the two Aqp1ab-type channels is that they functionally evolved in a manner that avoids competitive occupancy of the same plasma membrane space (see later).

Selective retention of *aqp1ab*-type genes in pelagophils

The existence of a second *aqp1ab*-type gene had until recently been overlooked, since species from all teleost cohorts, including eels, elephantnose fishes, zebrafish, salmonids, and soles, have either inactivated or lost one or both of the paralogs (Figure 2A). It was nevertheless revealed that the TSA1C likely arose in the common ancestor of teleosts >300 MA, but subsequently underwent a history of seemingly stochastic *aqp1ab*-type gene inactivation or loss [63]. However, the examination of >400 piscine genomes recently uncovered the almost exclusive retention of at least one paralog in pelagophils, with a third of the modern marine euacanthomorph pelagophils selectively retaining both copies (Figure 2B). By contrast, much higher fractions (>40% and >90%) of *aqp1ab*-type gene inactivation/loss, respectively, occurred in benthophils (see Outstanding questions) and species with internal egg incubation, such as rockfishes (Sebastidae family), seahorses, and pipefishes (Syngnathidae family). The selective retention of the *aqp1ab*-type genes thus seemed to be intuitively correlated with the degree of oocyte hydration (Figure 2C), yet a simple question arose: why do pelagophils need two water channels to perform the same function?

Molecular regulation of water channel trafficking

An initial answer to the previous question arose from observations that the *aqp1ab1* and *aqp1ab2* transcripts are highly enriched in the ovaries of marine pelagophils, but not those of freshwater benthophils [63,70], and that protein translation already occurs in primary stage oocytes before vitellogenesis [61,71,72]. Both water channel proteins were also observed in the plasma membrane of maturing oocytes with immunological inhibition of one of the channels only reducing the level of hydration by ~50% [61,71]. This suggested that in species that retain the complete TSA1C, both channels play essential roles in the hydration process. However, when attempting to investigate the molecular regulatory basis of channel intracellular trafficking in frog oocytes, the Aqp1ab2 paralog would not translocate to the plasma membrane, suggesting that trafficking mechanisms present in native teleost oocytes were absent in the frog gametes. Based on the combination of comparative transcriptomics, phylogenetics, and cellular expression systems, the essential factors that had neofunctionalized in relation to the frog repertoire were uncovered as two teleost-specific tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation proteins (**14-3-3 proteins**) zeta-like, termed 14-3-3ζLa and 14-3-3ζLb [63].

Box 3. Aquaporins: structure, regulation, and diversity

Aquaporins are integral membrane proteins found in all domains of life. Their discovery solved centuries of observations noting that water can traverse certain cell membranes more rapidly than the rate of diffusion and led to the Nobel Prize for Chemistry in 2003 [89]. Structurally, aquaporins are composed of six transmembrane domains linked by five loops (A–E) and two hemihelices bearing conserved Asn-Pro-Ala (NPA) motifs, with intracellular amino and carboxy termini (Figure I). They are assembled as tetramers, with the passive single-file conductance of water or other small, predominantly uncharged molecules, primarily determined in each monomeric pore by an aromatic-arginine (ar/R) selectivity filter, the NPA arrangement, and the transmembrane concentration gradient (see [58] for an overview of channel permeation preferences). The channels may be post-translationally glycosylated, or gated by protonation, Ca^{2+} binding, mechanical stress, and phosphorylation or dephosphorylation of selected residues in the loops and intracellular termini. Membrane trafficking regulation also occurs through phosphorylation/dephosphorylation of selected residues mainly in intracellular loops, and the N- and C-termini together with the attachment of binding proteins, or conversely via the expression of non-canonical inhibitory variants. Vertebrate aquaporin diversity consists of 17 subclasses (AQP0-16) phylogenetically separated into four grades of classical aquaporins, AQP8-type aquaporins, aquaglyceroporins, and unorthodox aquaporins [65]. Up to 15 of the subclasses have been identified in mammalian genomes, but they lack the AQP15 orthologs found in actinopterygian fishes, turtles, and crocodylians, and the AQP16 orthologs of amphibians, turtles, and crocodylians. Eutherian mammals also lack functional copies of AQP13 and AQP14 [90]. The genomes of diploid teleosts encode between 16 and 26 paralogs, depending on the lineage with the difference in copy number partly due to an additional whole genome duplication (WGD), but also a higher incidence of tandem duplication and gene loss [91]. Aquaporin gene clusters are consequently more prevalent in the teleost genomes compared with those of mammals (6:2) and include the recently discovered teleost-specific aquaporin-1 cluster (TSA1C) [63].

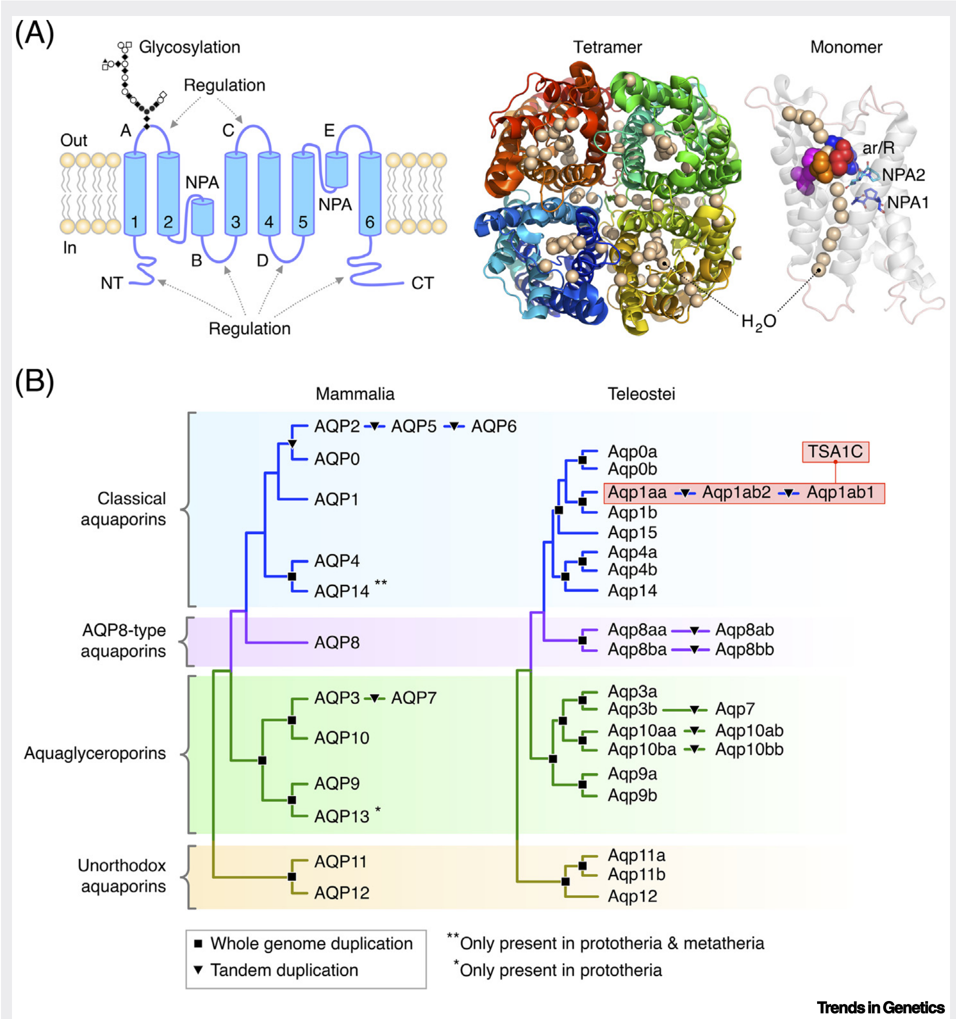

Figure I. Aquaporins: structure, regulation, and diversity. (A) Schematic illustration showing aquaporin transmembrane domains (1–6), the hemihelices bearing the Asn-Pro-Ala (NPA) motifs, the glycosylation site, and the regulatory subdomains in the amino- (NT) and carboxy- (CT) termini or loops (A–E). (B) Aquaporin tetramer (AQP5, PDB: 3D9S, [92]) showing water (spheres) traversing the central pore of each monomer. (C) Monomeric pore permeation is primarily determined by an aromatic-arginine (ar/R) selectivity filter and the NPA arrangement. (D) Water channel diversity in mammals and teleosts showing the likely mode of origin by gene duplication. Abbreviation: TSA1C: teleost-specific aquaporin-1 cluster.

Further investigations revealed that in pre-euteleost lineages that only retain the Aqp1ab1 ortholog, such as the cyprinid zebrafish, two protein kinase A (PKA)-mediated mechanisms evolved to post-translationally regulate its membrane trafficking, one dependent on the phosphorylation status of C-terminal Ser^{261} for preferential 14-3-3ζLa binding, and another controlled by Ser^{263} phosphorylation that is independent of the 14-3-3ζLa interaction [63]. In euteleostean salmonids and euacanthomorphs, however, only the C-terminal phosphorylation site for 14-3-3ζLa-binding is retained. In euacanthomorph pelagophils, the phosphorylation of this conserved site results in Aqp1ab1 trafficking to the oocyte cortex and partial translocation to the proximal region of the oocyte plasma membrane microvilli. By contrast, the euacanthomorph Aqp1ab2 channel requires phosphorylation of C-terminal Thr or Ser residues for the selective binding of

(A)

aqp1aa
aqp1ab2
aqp1ab1
Gilthead seabream
Atlantic halibut
Senegalese sole
Atlantic salmon
Zebrafish
Elephantnose fish
European eel
5 kb
④
③
②
①

(B)

Retain both *aqp1ab* genes
Lost both *aqp1ab* genes
Retain either *aqp1ab2* or *aqp1ab1*
aqp1ab-type gene retention
100%
75%
50%
25%
0%
5%
95%
33%
57%
43%
12%
91%
0%
9%
Pelagic [85]
Benthic [97]
Internal [35]

(C)

Intracellular H_2O
Extracellular H_2O
Embryo
Yolk
Yolk
~ 350 mOsm
~ 1000 mOsm
SW
VE
PVS
Embryo
Eggs
380
360
340
320
mOsm
Plasma
Pouch
Water content
100%
80%
60%
40%
***
93%
61%
***
78%
61%
*
51%
53%
Ooc
egg
Ooc
egg
Ooc
egg
Goldsinny wrasse
Corkwing wrasse
Greater pipefish

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(See figure legend at the bottom of the next page.)

14-3-3ζLb and membrane targeting (see Outstanding questions). It was then found that in pelagophils that maintain complete TSA1Cs, pituitary-secreted Lh and Avp, as well as local production of the nonapeptide in the ovarian follicles, activate both systemic and paracrine signal transduction cascades to co-operatively regulate the noncompetitive membrane trafficking of Aqp1ab-type channels in the oocyte and associated follicle cells [73].

Based on the gilthead seabream as an example of the mechanism, we have recently proposed a model to explain the neuroendocrine and paracrine regulation of aquaporin trafficking (see Outstanding questions) during oocyte hydration in euacanthomorph marine teleosts (Figure 3). In this model, neurohypophysial delivered Avp acts via an Avp receptor (Avpr2aa)-PKA pathway to induce the phosphorylation of Thr^{262} in the C-terminus of Aqp1ab2, facilitating the binding of 14-3-3ζLb and trafficking of the channel to the cell surface of the theca and granulosa cells. The maturational surge of adenohypophysial Lh couples with its cognate receptor (Lhcgr) to augment this mechanism in the granulosa cells. In addition, Lh and Avp stimulate the PKA-mediated synthesis of progestin in the granulosa cells, which then plays the role of a master switch, controlling the paracrine signal transduction of maturational GVBD and meiosis resumption in the oocyte via the membrane progestin receptor (mPgr), as well as the local granulosa cell production of Avp via the nuclear progestin receptor (nPgr). The latter synthesis of Avp further induces the paracrine signal transduction of the Avpr2aa-PKA pathway in the oocyte to phosphorylate Aqp1ab2 Thr^{262}, the binding of 14-3-3ζLb, and trafficking of the channel to the proximal region of the plasma membrane microvilli. The same Avp-Avpr2aa-PKA paracrine signaling pathway also causes phosphorylation of a conserved Ser^{156} in loop D of Aqp1ab1 that is pre-primed by 14-3-3ζLa binding, resulting in the membrane shunt of the channel to the distal region of the oocyte plasma membrane microvilli. Experiments then showed that the heterologous expression of both, rather than one of Aqp1ab-type channels, significantly increases the rate of bulk water uptake. Consequently, it seems plausible that gene retention of two closely related water channels that avoid competitive occupancy of the same plasma membrane space selectively evolved in euacanthomorph pelagophils by accelerating water influx during oocyte hydration. Such a feature would be compatible with the evolution of batch spawning in many marine teleosts, which requires several cycles of the hydration process, and improves the probability of survival [74].

In addition to the aforementioned signaling cascades controlling aquaporin trafficking, other regulatory mechanisms also occur, which may be more relevant to pelagophils that only retain one of the *aqp1ab*-type genes, such as soleid fishes [63]. Several nonfunctional splice variants of the Aqp1ab-type channels were shown to cause dual modes of **dominant-negative inhibition** of the canonical proteins [75]. One mode involves the likely hetero-tetramerization of Aqp1ab1 variants with the canonical channels and their retention in the endoplasmic reticulum (ER) for

Figure 2. Water channel genes are selectively retained in pelagophil teleosts. (A) Genomic organization of the teleost-specific aquaporin-1 cluster (TSA1C) in representative species of each cohort (1–4) (see Box 1 in the main text). † indicates inactivated pseudogenes. (B) Retention of *aqp1ab*1 and *aqp1ab2* genes is selectively related to the type of egg spawned. The number of euacanthomorph genomes screened is given in square parentheses. (C) Pelagic eggs hydrate intracellularly and develop a small extracellular perivitelline space (PVS) between the vitelline membrane and vitelline envelope (VE) when exposed to seawater (SW) (e.g., goldsinny wrasse, *Ctenolabrus rupestris* [53]). Intracellular hydration is lower in benthic eggs, which develop a large extracellular PVS (e.g., corkwing wrasse, *Crenilabrus melops* [53]). The oocytes (ooc) of species that incubate their eggs internally, such as seahorses and pipefishes, do not undergo major hydration, but subsequently develop a moderate PVS (e.g., greater pipefish, *Syngnathus acus*; Finn unpublished results). In this family, the male brood pouch (shown as cut-away with internal eggs) is hypo-osmoregulated in relation to SW and maintained close to isosmolality with the plasma during early embryonic development (e.g., Gulf pipefish, *Syngnathus scovelli* [12,13]). Statistical differences are indicated by $^{***}P < 0.001$; $^{*}P > 0.05$. See [35,85,97].

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Figure 3. Aqp1ab-type channel trafficking is differentially regulated to maximize oocyte hydration. The model is based on the euacanthomorph pelagophil gilthead seabream (modified from [63,73]). Circulating gonadotropins (luteinizing hormone, Lh) and nonapeptides (arginine vasopressin, Avp) secreted from the adenohypophysis and neurohypophysis, respectively, activate signal transduction cascades regulating germinal vesicle (GV) breakdown (GVBD), meiosis resumption and TSA1C-mediated oocyte hydration. C-terminal Thr^{262} of Aqp1ab2 is phosphorylated via a common Avp-Avpr2aa-protein kinase A (PKA) pathway, resulting in binding of 14-3-3ζLb and trafficking of the channel to the cell surface of the theca and granulosa cells. The activity of PKA in the granulosa cells is multiplied via the Lh-Lhcgr interaction, which also promotes the steroidogenesis (Stg) of the maturation-inducing progestin (17,20β-P). Paracrine signaling of progestin induces oocyte GVBD and meiosis resumption via the membrane progestin receptor (mPgr). Progestin also induces the granulosa cell production of Avp via the nuclear progestin receptor (nPgr), which acts as a paracrine signal to phosphorylate Aqp1ab2 Thr^{262} via the Avp-Avpr2aa-PKA pathway, targeting the channel to the proximal region of the oocyte plasma membrane

(Figure legend continued at the bottom of the next page.)

ultimate degradation, while the second allows Aqp1ab2 to partially escape from the ER to reach the oocyte plasma membrane, where water flux is dominantly-negatively inhibited through hetero-tetramerization with the nonfunctional **isoform** (Figure 3). Since developmental ovarian follicular expression levels of the inhibitory isoforms are lowest during meiosis resumption, an uninhibited water flux via the canonical channels in the oocyte microvilli prevails. In addition, a separate maturational PKC-mediated mechanism induces Aqp1aa recycling from the cell surface of the follicular epithelium [63,73]. This mechanism may prevent water efflux from the follicle while the uninhibited membrane insertion of the Aqp1ab-type channels in the theca, granulosa, and oocyte microvilli establish the hyperosmotic yolk as a one-way sink for the maternally imbibed water that is systemically delivered via the thecal capillary beds.

Finally, several regulatory mechanisms have been uncovered that leave the acquired water locked within the ovulated egg. Amongst these is the elevation of yolk pH towards neutrality at the end of the maturational cycle, indicating a reduction of H^+-ATPase and hydrolytic enzyme activity, and the cessation of yolk proteolysis [76]. Simultaneously, a **p38 MAPK**-mediated phosphorylation of a conserved residue located within the C-terminal 14-3-3ζL binding region of Aqp1ab1 in Euteleostei causes the release of 14-3-3ζLa, while 14-3-3ζLb is released from Aqp1ab2 by a different, as yet unknown, mechanism [63]. This results in recycling of the two water channels, which in some species may also be enhanced through the upregulation of the inhibitory splice variants [75]. These combined effects eliminate the yolk osmotic drive, which returns to isosmolality with the ovarian fluid (Figure 1D), and reduce the vitelline membrane water permeability, leaving the highly hydrated ovulated eggs ready for fertilization and their forthcoming sojourns in the oceanic currents.

Concluding remarks

The remarkable global diversification of modern euacanthomorph teleosts transpired predominantly in early Paleogene marine environments ~245 MA after the ancestral whole genome duplication (WGD) event at the root of the crown clade. The findings summarized in this review highlight *vtgAa* and *aqp1ab*-type water channel genes that arose by tandem duplication as founding contributors to the radiation of marine fishes. Their coevolution with 14-3-3ζ-like activation proteins led to key genetic adaptations that resulted in the developmental regulation of maturational yolk proteolysis and oocyte hydration. In their quest to reproduce in the extreme desiccating environment of seawater, such mechanisms initially provided the hyposmotic teleost embryos with the water of life, but subsequently caused their eggs to float. These permissive genetic adaptations are highly selectively conserved in euacanthomorph marine pelagophils and were thus 'seized upon' for the geodispersal and success of their eggs in the oceans.

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microvilli. The same paracrine Avp-Avpr2aa-PKA pathway stimulates phosphorylation of Ser^{156} in loop D of Aqp1ab1 that is pre-primed through a separate PKA-mediated phosphorylation of C-terminal Ser^{253} and preferential binding to 14-3-3ζLa, which results in translocation of the channel to the distal region of the oocyte plasma membrane microvilli. The differential localization of the Aqp1ab1 and Aqp1ab2 channels avoids competitive membrane space occupancy and accelerates bulk water influx. Dominant-negative inhibition of transmembrane water flux occurs via the expression of nonfunctional splice variants, which may either trap the canonical water channels in the endoplasmic reticulum (ER) or reduce the permeation rate across the cell surface. Dominant-negative mechanisms are developmentally downregulated to maximize oocyte hydration. A separate PKC-mediated phosphorylation of Aqp1aa, in response to a yet unknown signal, causes channel recycling in epithelial cells and likely establishes a one-way water flow to the hyperosmotic yolk.

Outstanding questions

Protein storage is a latency specialization of the normal endosome to lysosome degradation pathway, yet the mechanisms differentiating protein storage versus protein degradation remain mostly unknown. Can the elucidation of the pathways regulating the ancient mechanism of yolk protein storage and differential proteolysis shed light on the pathology of lysosomal storage dysfunction?

The ancestral process of yolk protein storage in oviparous vertebrates results in the formation of pseudocrystalline lattices. What is the molecular structure and purpose of the lattice and why does it not form in some species?

Water channels mediate oocyte hydration in pelagophils. Are they also involved in oocyte hydration in marine benthophils?

Aqp1ab-type channels mediate oocyte hydration in marine pelagophils, but also exist in some freshwater teleosts. What was the selective force favoring the retention of these genes in freshwater species?

The teleost-specific 14-3-3ζ-like activation proteins differentially control Aqp1ab-type trafficking during oocyte hydration. How are these binding factors regulated in the oocyte for this purpose and what are the molecular mechanisms involved?

Recent studies suggest that the rate of aquaporin endocytic recycling plays a potentially dominant role controlling channel function and plasma membrane permeability. Is this rate mechanism also important in the trafficking regulation of Aqp1ab-type channels during oocyte growth and hydration?

Oocyte hydration is one of the most critical events in the life cycle of many marine teleosts, since failure of the eggs to float results in negative selection. How will climate change impact this mechanism and the biodiversity of so many pelagophil species?

Can genetic variations in the TSA1C or in the oocyte hydration pathways serve as biomarkers to evaluate and predict egg viability?

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Declaration of interests

The authors declare no competing interests.

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