Metal Ion Fluxes Controlling Amphibian Fertilization

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Abstract

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Mammalian oocytes undergo major changes in zinc content and localization in order to be fertilized, the most striking being the rapid exocytosis of over ten billion zinc ions, known as zinc sparks. Here we report that fertilization of amphibian *Xenopus laevis* eggs also initiates a zinc spark that progresses across the cell surface in coordination with dynamic calcium waves. This zinc exocytosis is accompanied by a newly recognized loss of intracellular manganese: synchrotron-based X-ray fluorescence and analytical electron microscopy reveal that zinc and manganese are sequestered in a system of cortical granules that are abundant at the animal pole. Through Electron-Nuclear Double-Resonance (ENDOR) studies, we rule out Mn^{2+} complexation with phosphate or nitrogenous ligands in intact eggs but the data are consistent with a carboxylate coordination environment. Our observations suggest that zinc and manganese fluxes are a conserved feature of fertilization in vertebrates and that they function as part of a physiological block to polyspermy.

Graphical abstract
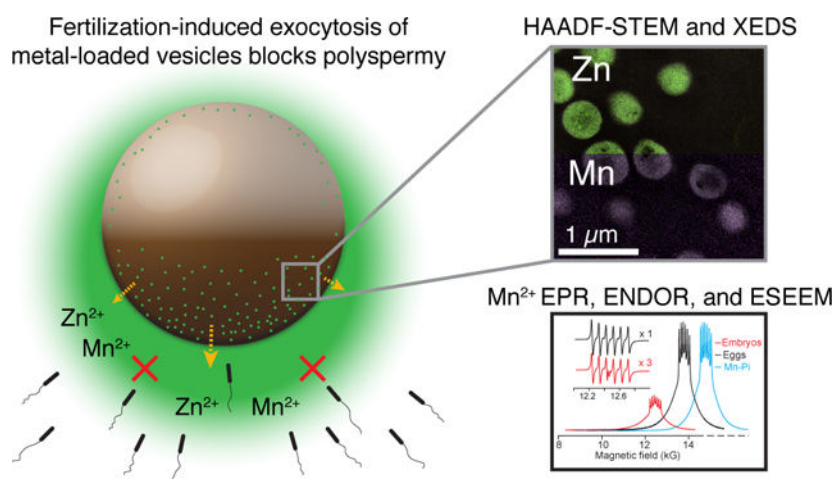
Introduction

Physiochemical approaches are opening the door to understanding how inorganic chemistry regulates key biological processes. A striking example of this is the discovery of zinc regulatory pathways controlling fertilization of mammalian oocytes which begins with the coordinated uptake of billions of zinc ions in the brief final stages of meiotic maturation.[1] Upon fertilization, murine eggs then rapidly release between 10–20% of their zinc from a pool of 8,000 cortical vesicles in events described as zinc sparks.[2,3] First observed using zinc responsive fluorescent probes in *M. musculus*[2], these events occur in macaques,[2] *B. taurus*[4] and *H. sapiens*[5] and are essential for transition of the egg into an embryo. Studies of mouse eggs reveal several roles of these zinc sparks: they regulate transitions from the meiotic to mitotic cell cycle of the egg and resulting zygote, and play roles in the slow block to polyspermy in the cell envelope.[6,7] The magnitude of the zinc spark, readily established in real time using fluorescent probes, correlates with the quality of the resulting embryo[8,9] Analogous zinc-dependent phenotypes are oberved in *Caenorhabditis elegans*[10] and *Drosophila melanogaster*[11], where zinc availability is required for embryo viability.

While eggs from each of these provide specific advantages, each is limited by size and low material yields. This is not the case for the African Clawed Frog, *Xenopus lavis*.

While zinc is known to be transported into *Xenopus* oocytes over a period of months during oogenesis[12,13] little is known about whether a zinc cycle occurs in this or other amphibians: neither influx nor exocytosis of zinc has been reported during meiosis. We decided to test whether meiotic zinc transients occur in *Xenopus* because of the large size and the abundance of eggs produced by the frog. The diameter of *Xenopus* eggs is fifteen times that of mouse eggs, allowing for a more in-depth study of the dynamics of the zinc efflux. While the abundance of auto-fluorescent yolk platelets in frog eggs presents challenges in confocal microscopy studies, the large size and the fact that a typical frog extrudes hundreds of eggs at a time, each with an average dry weight of 0.45 mg/egg,[14] allows for the application of methods such as Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and Electron-Paramagnetic Resonance (EPR) that otherwise would require an formidably large number of mammalian eggs. These bulk-sample methods provide key benchmarks and allow for our application of an array of more sensitive quantitative methods for understanding the chemistry of metal ion fluctuations in the regulation of biological processes at both the single cell and subcellular level.

Here, we present several complimentary physical methods that show that fertilization triggers release of zinc from vesicular storage compartments at the surface of the frog egg. These cortical vesicles, located predominantly at the animal pole, are the source. Our results suggest that the storage of labile zinc complexes and their triggered release as zinc sparks may be an evolutionarily conserved and functional feature of fertilization from amphibians to mammals. Intriguingly, *Xenopus* differs from mammals by also concentrating manganese in cortical vesicles and releasing a significant amount of the egg's Mn^{2+} content at the time of fertilization. We find that extracellular Zn^{2+} or Mn^{2+} decreases the rate of *Xenopus* fertilization, suggesting that the triggered release of these metal ions contributes to blocking the entry of a second sperm which would be fatal to a developing embryo. The paramagnetism of Mn^{2+} allows us to glean insights into the local coordination environment of manganese within the cortical granules in ways that are not possible for its d^{10} congener Zn^{2+}. These chemical and functional insights into the inorganic chemistry of an amphibian egg expand the evolutionary context of zinc, and possibly now manganese, fluctuations as signaling events in biology.

Results

The zinc spark is conserved in amphibia

Live cell fluorescent microscopy experiments using the fluorescent zinc-responsive probe FluoZin-3 were employed to test whether zinc is released from *Xenopus* eggs upon fertilization or activation by chemical treatment. Following the addition of sperm, a single zinc release event is observed. Moreover, beginning at a single point on the oocyte surface, zinc release proceeds bidirectionally around the circumference of the egg, taking around 3 minutes to reach the opposite point (Figure 1A, Video 1). The zinc spark in mammalian oocytes has been shown to be a calcium-dependent exocytosis event, and the Ca^{2+} ionophore ionomycin is known to parthenogenetically activate amphibian eggs.[15]

Using these conditions as a guide, the addition of 20 μM ionomycin to frog eggs leads to a single rapid and intense release of zinc (Figure 1B, Video 2). The zinc efflux induced by addition of ionomycin starts at a broad section of the circumference and travels around the circumference of the egg within approximately 30 – 90 seconds.

Upon fertilization, the zinc efflux travels approximately linearly around most of the circumference of the egg at 9.7 ± 0.3 μm/sec (n = 3, mean ± SD). Figure 1C and D show the rate of efflux around the circumference (see Supplemental Figure 1 for procedure). Fertilization is known to initiate a well-established biochemical cascade leading to calcium release from intracellular stores.[16] This is apparent in the calcium wave, which travels at an average of 8.9 μm/s[17], similar to that of our observed zinc efflux. The calcium wave starts at the site of sperm entry, and travels around the egg in approximately 3.5 minutes[17], about as long as the zinc spark, suggesting that the calcium wave triggers zinc release. Zinc release in eggs activated by ionomycin is more rapid, likely because ionomycin is bringing calcium indiscriminately to all parts of the egg simultaneously, leading to a faster release of cortical granule (CG) content. Zinc is known to be a cofactor for the ZPA protease, which is released from *Xenopus* eggs and hardens the ZPA glycoprotein following fertilization, contributing to the block to polyspermy.[18] Given that the zinc-binding affinity of FluoZin-3 (K_D 9.1 nM)[19] is lower than that of most known zinc proteins,[3,20] the observed extracellular fluorescence signal is likely arising from free zinc.

The change in intracellular distribution of this cofactor resulting from zinc efflux can be mimicked by treatment with intracellular metal chelating agents. In mouse eggs, treatment with these agents leads to parthenogenic egg activation supporting the conclusion that zinc efflux is necessary for activation.[2] To test whether this is true in frog as well as in mammals, *Xenopus* eggs were treated with cell-permeable chelators and scored for activation. Treatment with N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine [TPEN] does not activate frog eggs, probably due to its low solubility; however, treatment with 1,10-phenanthroline (OP) activates $97 \pm 5\%$ of the eggs (Mean ± SD, n = 3. See Supplemental Figure 2, Videos 3 – 5). To test whether OP causes activation through lowering intracellular copper availability, eggs were treated with ammonium tetrathiomolybdate (TTM), which has a high selectivity and specificity for copper.[21] TTM did not activate the eggs (Video 6) indicating that activation is driven by a decrease in zinc, instead of a decrease in copper availability. The absence of validated intracellular chelators that are specific for other transition metals limits our ability to address their roles in the activation of mammalian or amphibian eggs.

Fertilization-induced changes in metal content

While the zinc content of mouse eggs drops circa 20% upon fertilization, a previous study using atomic absorption spectroscopy revealed no difference in the zinc content of single *Xenopus* eggs and embryos.[13] To determine whether zinc content changes immediately following fertilization, we analyzed eggs and embryos using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). We compared eggs to embryos collected one-hour post-fertilization and found no statistically significant change in zinc content (Figure 2A).

Fluorescence optical microscopy nonetheless clearly indicates that zinc is released upon fertilization; we hypothesize that this release corresponds to only a small percentage of the total zinc contained in these large eggs. In contrast to mammalian eggs, at the time of fertilization approximately 90% of intracellular zinc in *Xenopus* eggs is found in the yolk.[12,22] As discussed in detail below, it is not surprising that only a very small proportion of intracellular zinc ions is released.

We also determined concentrations of other transition metals before and after fertilization. There was no change in the average iron or copper concentration following fertilization. However, intracellular manganese decreased by $53 \pm 15\%$ (n = 4, $\pm$ SEM) (Figure 2A). We did not detect changes in other elements (Figure 2B and Supplemental Table 1 for values in atoms/cell). As this was an unexpected finding, we decided to probe it further.

The coordination environment of manganese

Intracellular manganese is predominantly present in the +2 oxidation state.[23,24] The majority of that is found in kinetically labile complexes with low molecular-weight metabolites,[25–28] but it is also bound as a cofactor in a number of enzymes.[29] Mn^{2+} is paramagnetic (S=5/2), and we employ a combination of Electron-Paramagnetic Resonance (EPR), Electron-Nuclear Double-Resonance (ENDOR) and Electron Spin-Echo Envelope Modulation (ESEEM) spectroscopies, complementary means of obtaining an NMR spectrum of the ligands to a paramagnetic metal ion (see Supplemental Methods), to determine the relative populations of the different Mn-ligand complexes present in intact eggs before and after fertilization.[25,26,28]

The absorption-display continuous-wave (CW) EPR spectrum of frozen intact eggs and embryos provides a well resolved six-line pattern created by the hyperfine interaction of the electron spin with ^{55}Mn nuclear spin ($I = 5/2$). This pattern is centred around magnetic field of ~12kG, and rides on relatively narrow "wings" (Figure 3A and see SI); the latter are suppressed in derivative-mode CW EPR spectra (inset). This pattern is typical of Mn^{2+} coordinated in high-symmetry (roughly octahedral) geometry, as seen for small Mn-metabolite complexes like Mn-phosphate (Mn-Pi). Broad "wings" associated with asymmetric Mn^{2+} chelate and enzyme environments (i.e. MnSOD)[27,28] are absent in the spectra of *Xenopus* eggs and embryos. The shape of the Mn^{2+} EPR spectrum of the embryos is unchanged from that of the eggs (Supplemental Figure 3) but the amplitude of the embryo signal is about one-third that of the eggs (~30 $\pm$ 10% remaining; see Figure 3, legend, and Supplemental Methods). Given that the spectra have the same shape, their relative amplitudes are an appropriate measure of Mn^{2+} present. The decrease in EPR amplitude upon fertilization agrees with the loss of Mn measured by ICP-MS (Figure 2), within the mutual uncertainties of the measurements, together showing that the egg loses Mn^{2+} upon fertilization.

ENDOR and ESEEM were used to determine the intracellular speciation of the Mn^{2+}. Figure 3B shows 35 GHz pulsed ENDOR spectra of eggs and embryos (triplicates) and selected exemplar Mn^{2+} complexes (hexa-aquo Mn^{2+} and the Pi, and polyphosphate [polyP]). The $[Mn-(H_2O)_6]^{2+}$ sample shows a strong ^1H ENDOR response with multiple peaks centered at the ^1H Larmor frequency (shown under the ^1H 'brace') assigned to

directly-coordinated water molecules.[30] When a metabolite moiety such as a phosphate binds to Mn^{2+} it replaces coordinated water(s), decreasing the ^1H ENDOR amplitude. When a phosphate binds, this also introduces a ^{31}P ENDOR doublet, split by the electron-nuclear interaction and centered at the ^{31}P Larmor frequency (shown under the 'brace' ^{31}P). In Mn-polyP complexes, multiple phosphates bind, most of the waters are displaced, as shown by the large reduction of ^1H ENDOR signal, and there is an even greater increase in ^{31}P ENDOR signal (Figure 3B). When the amplitudes of the ^{31}P, ^1H ENDOR peaks shown in Figure 3B as gray stripes are normalized to the corresponding Mn^{2+} concentrations they are denoted as absolute ENDOR responses and labelled as $^{31}P\%$, $^1H\%$. These responses can be used in a heuristic model of Mn^{2+} ligand binding described previously[26] to estimate fractions of aquo, Pi, polyP, and ENDOR-silent (e.g. carboxylate) complexes (see SI).

In comparison to the exemplars, both eggs and embryos show weak ^{31}P signals (inset a), indicating little Pi/polyP ligation, and a weak $^1H\%$ ENDOR response (inset b), yet indicating a high population of ligands that displace H_2O. To test for Mn^{2+} ligation by nitrogenous ligands we used 35 GHz ESEEM spectroscopy, which is a very sensitive probe towards such ligation[25–28]; Figure 3C shows that the ESEEM time-wave for a frozen solution of Mn^{2+}-imidazole exhibits strong modulation whose frequency corresponds to ^{14}N resonance frequencies. Neither eggs nor embryos show any ^{14}N modulation of the time-waves in the 35 GHz ESEEM traces, indicating the absence of any detectable Mn-N complexes. Together, these observations indicate that the majority of the intracellular Mn^{2+} coordination sites (i.e. between 75–90%) in both eggs and embryos are occupied by ENDOR (NMR)-silent ligands, and when an organism is grown with natural abundance ^{12}C nutrients, carboxylates are the major class of such ligands. We conclude that the majority Mn^{2+} population in the eggs and embryos can be assigned as low molecular-weight Mn^{2+} complexes with carboxylate ligands (Mn-C 75–90%).

Disruption of eggs exposes compartmentalized Mn^{2+} complexes to cytoplasmic concentrations of phosphate and phosphoryl ligands which are typically in the millimolar concentration range.[31] As a result, scrambling induces a 20% increase in the Mn-P population (Supplemental Figure 3 and Supplemental Methods). Taken together, the ENDOR measurements are consistent with a model in which a majority of the EPR-detectable Mn^{2+} is sequestered as manganese-carboxylate in non-cytosolic compartments in the egg, and that these complexes are released from the egg upon fertilization without change in speciation of the remaining Mn^{2+}. To test for the presence of Mn- and Zn-enriched compartments in these eggs we turned to two subcellular methods for mapping the locations of elements in cells.

XFM reveals cortical metal compartmentalization

X-ray Fluorescence Microscopy (XFM)[32] methods indicated that cortical vesicles in mouse eggs contain on average one million zinc ions each[3] and we employed this approach to test for the presence of analogous zinc- and manganese-enriched sites in small sections at the cortices of *Xenopus* eggs and embryos. To preserve transition metal contents in our samples we used a modified form of Timm's silver staining.[3,33,34] ICP-MS reveals that this fixation method preserves native metal contents in *Xenopus* eggs, except for iron, which showed

a significant gain in concentration, due to contamination in the fixative (Seeler et al., in preparation). We analyzed ultramicrotome slices of the animal pole (AP) and vegetal pole (VP) of eggs and embryos, ranging from 0.4 to 2 μm thick. The cortical ultrastructure of the AP and VP are greatly different.[35] We find that metal content and localization differ in the poles as well (Figure 4 and Supplemental Figures 4 and 5). We conclude that in *Xenopus* eggs, zinc is loaded to high concentrations in the smaller group of cortical vesicles that are analogous to those found in murine, bovine and primate eggs.[2,4,5]

We find two classes of metal-containing compartments: the smaller ones are far more abundant in the AP than the VP. Fitting the XFM data reveals that metals are localized in compartments at millimolar-scale concentrations. Figure 5A presents the XFM values as μg/cm^2 next to an estimate of the minimal molar concentration in each site. The conversion to mM was obtained through estimates of the number of atoms in each voxel, as the sample thickness is known (see SI for more detail). There is some variation in the metal content of these compartments, though some of this is because slices of eggs were analyzed, so compartments may represent sections or combinations of what we assume to be vesicles (Figure 5B and C). There is metal concentration in these compartments: Mn concentration is approximately 30X higher in the compartments compared to the entire volume of the egg (see Figure 2B), Cu is 150X, and Zn is 10X.

The second class of metal-containing compartments is larger and contains a lower concentration of metal. These are likely yolk platelets. Yolk consists of two proteins: lipovitellin, which binds zinc, and phosvitin, which contains 25% phosphoserine.[36] These compartments are also larger in the VP cortex than the AP,[37] further supporting this assignment. Besides zinc and to an extent copper, transition metals are at very low concentrations in the yolk platelets (Supplemental Table 2).

Unlike mammalian eggs, we find other metals in addition to zinc localized to the *Xenopus* egg cortex. To determine the extent of metal co-localization, Pearson coefficients were calculated. The egg AP sections had the greatest amount of correlation with low variability (Supplemental Figure 6). Two main elemental groupings with high Pearson coefficients (0.64 – 0.92) are observed. The first is cobalt, copper, and nickel, and the second is calcium, manganese and zinc. A possible source of metals with little or no known biological role in vertebrates (e.g. cobalt and nickel) is the water in which the animals are housed. Water from frog tanks was analyzed by ICP-MS and transition metals were found to be at approximately nanomolar concentrations (Supplemental Table 3).

While XFM provides very high elemental sensitivity, the spatial resolution at 2-ID-D (300 × 300 nm pixels) does not allow for high resolution imaging of the metal-enriched sites. Thus, additional high-resolution elemental mapping methods were employed to characterize these sites.

AEM demonstrates metal vesicular localization

To assess whether these sites represented metal-enriched vesicle structures, nanometer resolution images were obtained using state-of-the-art Analytical Electron Microscopy (AEM) which combines Scanning Transmission Electron Microscopy (STEM) with X-ray

Energy Dispersive Spectroscopy (XEDS).[38] Images were obtained using 150 nm-thick sections of eggs and embryos. Similar to the XFM data, AEM revealed two classes of vesicle, with the smaller ones containing multiple metals, and the larger ones containing primarily phosphorous and sulfur, with very low concentrations of the other elements examined (Figure 6A and B). Ca, Mn, Co, Ni, Cu, and Zn are found in the smaller class of electron-dense vesicle (Figure 6C). The average diameter of the small vesicle in the egg AP is 490 ± 56 nm (mean ± SD, n = 50) (Figure 6D). These images concur with our XFM findings. There are fewer small vesicles in the animal pole embryo than in the egg (Supplemental Figure 7). Both the vegetal pole egg (Supplemental Figure 8) and vegetal pole embryo (Supplemental Figure 9) show large yolk platelets and few of the small vesicles.

Using TEM images of sections of the circumferences of an egg and embryo AP, we counted the numbers of small, electron-dense vesicles in the cortex. Within 10 μm of the plasma membrane, there was an average of 21 vesicles/100 $μm^2$ in the egg, compared to an average of 3.4 vesicles/100 $μm^2$ in the embryo, corresponding to an 84% drop (Supplemental Figure 10). We can therefore conclude that we see an almost complete release of cortical vesicle content. Additionally, this analysis suggests that other vesicular metals beside zinc and manganese are released from the zygote following fertilization. Finally, we sought to determine the biological relevance of this metal release.

Treatment with Zn or Mn decreases the fertilization rate

To determine if the zinc and/or manganese released from *Xenopus* eggs following fertilization prevents polyspermy, we fertilized eggs in buffer containing different concentrations of the two metals. Increasing concentrations of both zinc and manganese (Figure 7, Supplemental Table 4; see Supplemental Figures 11 and 12 for raw data) lead to decreasing rates of fertilization. This was determined by the percent of eggs that become properly-cleaving embryos (after normalization to a control fertilization), divided by the total count of eggs, embryos, and failed cleavage (Figure 7A). Zinc inhibits fertilization at an order of magnitude lower concentration than that of manganese: a 50% block to fertilization occurs at 53 μM (95% CI = 47 – 61 μM) in $ZnSO_4$ treatments (Figure 7B), while it occurs at 890 μM (95% CI = 770 μM – 1.0 mM) in the case of $MnCl_2$ (Figure 7C).

Discussion

The goal of this study was to determine whether zinc is aggregated in a system of vesicles near the surface of *Xenopus* eggs and then released in a highly coordinated manner, similar to what has been shown for mammalian model organisms and human eggs. Indeed, we show that *Xenopus* eggs, like mammalian ones, undergo zinc-chelator induced activation, contain a similar system of zinc-loaded vesicles, albeit larger than those seen in mammalian eggs,[3] as well as demonstrate an efflux of zinc following activation and fertilization. Thus, these reorganizations of zinc are essential to successful development in mouse and *Xenopus*. We demonstrate that one function of the zinc released acts as part of a block to fertilization in *Xenopus*: the entry of a second sperm is typically catastrophic in early embryonic development. Further experiments are necessary to determine if, as in mouse eggs, zinc

alters the structure of the outer envelope in *Xenopus* eggs. It is possible that release into the jelly coat could have an effect as well.

Surprisingly, we find that manganese is released alongside zinc in *Xenopus* eggs. Manganese, as well as a series of non-essential transition metal ions, is stored in vesicular compartments. Several lines of reasoning support the conclusion that the cohort of small metal-containing vesicles describe here are CGs. First, the timing of the zinc spark in *Xenopus* eggs correlates directly to the well-established process of CG breakdown. CG breakdown begins around 3 minutes post-fertilization (approximately the time needed for the sperm to penetrate the egg) and virtually all CGs have ruptured within 10 minutes.[35,39] Second, the spatial distribution of CGs resembles that of the metal-containing compartments. CGs in the AP are mostly next to the plasma membrane, while those in the VP can be located farther into the cortex.[35] Third, the reported sizes of CGs – 1.5 – 2.5 μm[35,40] – are similar to those of the metal-containing compartments. Although the vesicles described here appear smaller than the literature values, this is likely an effect of fixation and/or treatment with sodium sulfide. The zinc-containing CGs of mouse eggs are approximately 260 nm in diameter and have an intra-vesicular zinc content of 0.2 M.[3] *Xenopus* CGs are therefore larger but have lower zinc concentrations.

The role of cobalt and nickel loading in *Xenopus* CGs is currently unknown. Because of their toxicity, these metals may be sequestered in the egg CGs to minimize teratogenicity. It is also possible that the metals are not intentionally localized to CGs, but rather are mistaken for Zn^{2+} and Mn^{2+} by promiscuous divalent metal transporters. Sequestration and release of these metals may be specific to amphibians; we will further explore this in mouse.

These results in *Xenopus* provide evidence of the Mn^{2+} coordination environment in an egg. While the EPR and ENDOR signals drop as expected upon fertilization, the speciation of Mn remaining after Mn release at fertilization remains largely unchanged. Most importantly, both eggs and embryos have only a minimal population of Mn-Pi and polyP complexes (~10%), with most Mn carboxylate-bound (75–90%), and with negligible amounts bound in high molecular weight proteins/enzymes such as MnSOD. The coordinating carboxylates are presumed to be associated with low molecular weight metabolites. This type of insight into the manganese coordination environment in small subcellular compartments provides a starting point for understanding the chemistry of other metal ions in these highly specialized compartments.

Manganese content of eggs was not directly determined in our studies of mouse, worm, and cow as yields of eggs in these systems are too low to analyze with ICP-MS. Previously published single cell data on mouse eggs was acquired on XFM beamlines that did not provide sufficient incident flux for detection of manganese signals; however the single cell studies described here open the door to the evaluation of these questions. While the role of the manganese efflux is not known, one possible role may be as a block to polyspermy. In a mouse model, Mn^{2+} inhibited sperm-zona pellucida interactions, with 50% inhibition at 1.25 mM $MnCl_2$.[41] This is similar to what we found in our *Xenopus* model, in which half of fertilization was inhibited at 890 μM $MnCl_2$. Estimating the concentration of Mn released in the area around the *Xenopus* zygotes gives a value in the hundreds of micromolar range (see

SI for calculations). Thus our results are consistent with a model in which the Mn and Zn release functions in part to block the entry of a second sperm, though additional tests of this mechanism are required.

In conclusion, we have found that a zinc exocytosis event upon fertilization is evolutionarily conserved between mammals and amphibians. Additionally, we have found that manganese is also released from *Xenopus* eggs and that both metals can inhibit fertilization.

Methods

Additional details including descriptions of methods and calculations are included in Supplemental Information.

Confocal Imaging.

Xenopus eggs were obtained via established methods. Eggs were acquired from female *Xenopus laevis* (Nasco) between the ages of 3 to 10 years old. Confocal images were taken at 2.5X using a Leica SP5 microscope (Biological Imaging Facility, Northwestern University). Eggs were imaged in 0.1X Ca^{2+}, Mg^{2+}, and EDTA-free Marc's Modified Ringer's (MMR: 10 mM NaCl, 200 μM KCl, 500 μM HEPES pH 7.4) buffer containing 50 μM FluoZin-3 tetrapotassium salt ((ThermoFisher Scientific).and implement XFM experiments and process and analyze) In order to parthenogenically activate the eggs, ionomycin calcium salt from *Streptomyces conglobatus* (Sigma Aldrich) in DMSO was added to the buffer at a final concentration of 20 μM. To image fertilization, around half of a testis was ground in 200 μl 1X Ca^{2+}, Mg^{2+}, and EDTA-free MMR (100 mM NaCl, 2 mM KCl, 5 mM HEPES pH 7.4) and kept on ice. Before imaging, the sperm solution was mixed 1:1 with 0.1X Ca^{2+}, Mg^{2+}, and EDTA-free MMR and added at a 1:10 dilution to the buffer in the imaging dish.

ICP-MS.

The jelly coat of eggs and embryos was removed through soaking in a 3% w/v solution of cysteine-HCl, pH 8 for 10 minutes. They were rinsed and then 20 egg/embryos were placed in a metal-free conical tube. 3–5 tubes of eggs/embryos were analyzed for each of the 4 separate frogs. The eggs/embryos were dried in a heat block and dissolved in 450 μl 67–70% trace-metal free nitric acid (Supelco) overnight at 70 C. The solution was diluted to 15 ml using MilliQ H_2O. Metal concentrations were measured using a Thermo iCAP Q ICP-MS and a Thermo iCAP 7600 ICP-OES (Quantitative Bio-element Imaging Center, Northwestern University). The elemental composition of frog tank water was measured using a Thermo iCAP Q ICP-MS and a Thermo Element2 High Resolution Magnetic Sector Field ICP-MS (Quantitative Bio-element Imaging Center, Northwestern University)

EPR/ENDOR/ESEEM Spectroscopy.

Frog eggs and embryos were loaded into a quartz EPR sample tube, flash-frozen in liquid nitrogen, and stored at 77 K until analysis. 35 GHz continuous-wave (CW) EPR spectra were recorded using a lab-built 35 GHz EPR spectrometer.[42] Absorption-display EPR spectra were collected from eggs/embryos and Mn-metabolites by using CW "rapid

passage" methods at 2 K as previously described.[25,27] Pulsed ENDOR/ESEEM spectra were recorded using a lab-built 35 GHz pulsed EPR spectrometer.[43] All spectra were recorded at 2 K using an immersion helium cryostat. Experiments were performed on three sets of samples and the average $^{31}P\%$, $^{1}H\%$ were used to calculate the fractional Mn^{2+} speciation. The fractional populations of Mn-L, L = H_2O, Pi, polyP, and ENDOR-silent complexes reported as percentages were obtained using a heuristic binding model as previously described.[26]

Sample Fixation and XFM.

Eggs/embryos were fixed in a solution containing NaSH (2.5% glutaraldehyde, 2% paraformaldehyde, 20 mM NaSH, in 0.1 M PIPES buffer, pH 7.4) for 2 hours at RT. They were then dehydrated in EtOH. After dehydration, eggs/embryos were infiltrated with a Durcupan resin and polymerized at 65° C for 24 hours. Sections of 400 nm and 2 μm thickness were obtained with a Ultracut-S ultramicrotome (Leica) using a diamond knife (Diatome/EMS). Egg and embryo sections were mounted on 200 nm-thick silicon nitride windows (Norcada) and were glued to aluminum sample holders using clear nail polish. The sections were imaged at Beamline 2-ID-D at the Advanced Photon Source at Argonne National Laboratory.

Analytical Electron Microscopy.

Eggs and embryos used in AEM studies were fixed as above. ~ 150 nm thick ultramicrotome-cut sections were mounted on an aluminum grid coated with a Formvar and a carbon film and mounted in a custom-manufactured beryllium-tipped cryo-transfer tomography holder from Fischione Instruments. Experimental measurements were performed using a ThermoFisher Talos F20 and the prototype ANL PicoProbe AEM's, both equipped with the most recent generations of high solid angle (~ 1sR) X-ray energy dispersive spectroscopy systems. All observations were made at 200 kV in both TEM and STEM modes at room temperature. Temporally resolved hyper spectral imaging (HSI) was used to scan variable area regions of interest at spatial resolutions ranging from 2–100 nm. Probe currents employed were typically ~ 100 pA, HSI regions of interest utilized pixel dwell times of ~ 25 μsec/pixel for hundreds of frames to develop statistics and mitigate drift, resulting in final data acquisition times of ~ 500 msec/pixel. Programs used for data acquisition and analysis were ThermoFisher Scientific TIA/ESVision and ThermoFisher Scientific Velox.

Fertilization Experiments.

Eggs were placed in 0.1X EDTA-free MMR containing different concentrations of $ZnSO_4$ or $MnCl_2$ for 15 minutes. They were fertilized using a sperm solution containing the same metal concentration. 15 minutes after fertilization, the dishes were flooded with the corresponding metal solutions. After cleavage became apparent, eggs were scored between uncleaved, properly cleaved, and failed cleavage. The percent of eggs properly cleaved in the experimental groups was normalized to that of a control group fertilized in buffer that was not spiked with zinc or manganese.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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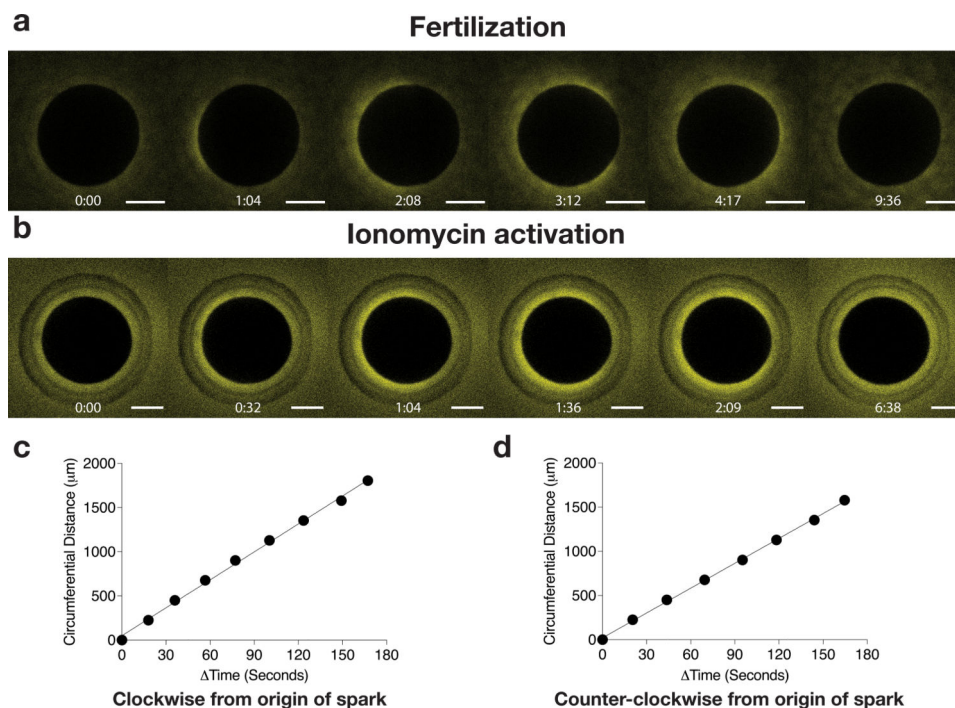
Figure 1: Zinc release from frog oocytes.
Zinc is released following both fertilization and chemical activation of a *Xenopus* egg.
a. Zinc efflux following fertilization of a *Xenopus* egg. Images are representative of 14 eggs from 4 separate frogs over 4 independent experiments. **b.** Zinc efflux following parthenogenic activation of a *Xenopus* egg by ionomycin. Images are representative of 12 eggs from 3 separate frogs over 3 independent experiments. In both **a.** and **b.** scale bar = 500 μm, time is as m:ss, and brightness and contrast have been adjusted for clarity (see Supplemental Methods for values). **c.** Representative plot of the circumferential distance from the origin of the zinc spark at which half-maximal fluorescence is measured over the time since the start of the spark, traveling clockwise from origin. **d.** Representative plot of the circumferential distance from the origin of the zinc spark at which half-maximal fluorescence is measured over the time since the start of the spark, traveling counterclockwise from origin. **c.** and **d.** are representative of measurements from 3 separate eggs (see text for mean rate).
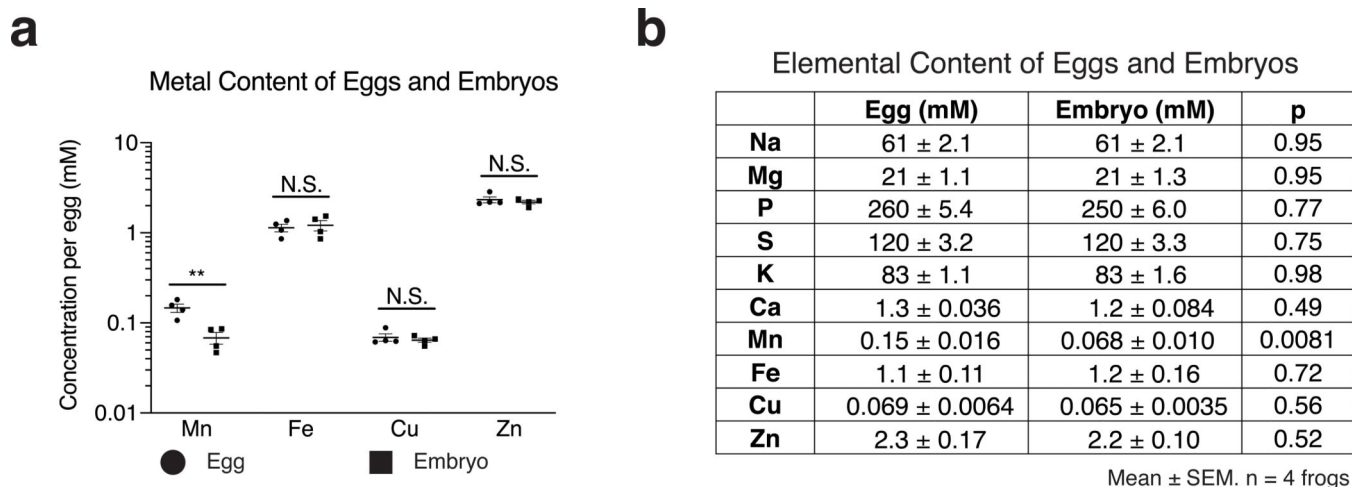
a

Metal Content of Eggs and Embryos

b

Elemental Content of Eggs and Embryos

	Egg (mM)	**Embryo (mM)**	**p**
Na	61 ± 2.1	61 ± 2.1	0.95
Mg	21 ± 1.1	21 ± 1.3	0.95
P	260 ± 5.4	250 ± 6.0	0.77
S	120 ± 3.2	120 ± 3.3	0.75
K	83 ± 1.1	83 ± 1.6	0.98
Ca	1.3 ± 0.036	1.2 ± 0.084	0.49
Mn	0.15 ± 0.016	0.068 ± 0.010	0.0081
Fe	1.1 ± 0.11	1.2 ± 0.16	0.72
Cu	0.069 ± 0.0064	0.065 ± 0.0035	0.56
Zn	2.3 ± 0.17	2.2 ± 0.10	0.52

Mean ± SEM. n = 4 frogs

Figure 2: Elemental analysis of eggs and embryos.

ICP-MS measures a significant decrease in intracellular manganese post-fertilization. **a.** Metal concentrations of *Xenopus* eggs and embryos (1 hour post-fertilization) determined by ICP-MS. Intracellular Co and Ni are both below 13 μM. Data are presented as mean ± SEM per egg/embryo, **: **p** = 0.0081. Each point corresponds to ICP-MS analysis of a batch of 20 eggs normalized to a per egg basis. Similar results were obtained in 6 separate experiments (not shown). **b.** Elemental contents (mM) of *Xenopus* eggs and embryos (1-hour post-fertilization). Experimental conditions as in **a**. In both **a.** and **b.** n = samples from 4 separate frogs analyzed in a single ICP experiment. **p** is the significance of the change in elemental content following fertilization. Two-tailed, heteroscedastic T-tests were run between eggs and embryos in order to determine if there was a significant difference in metal content. No adjustments were made for multiple comparisons.
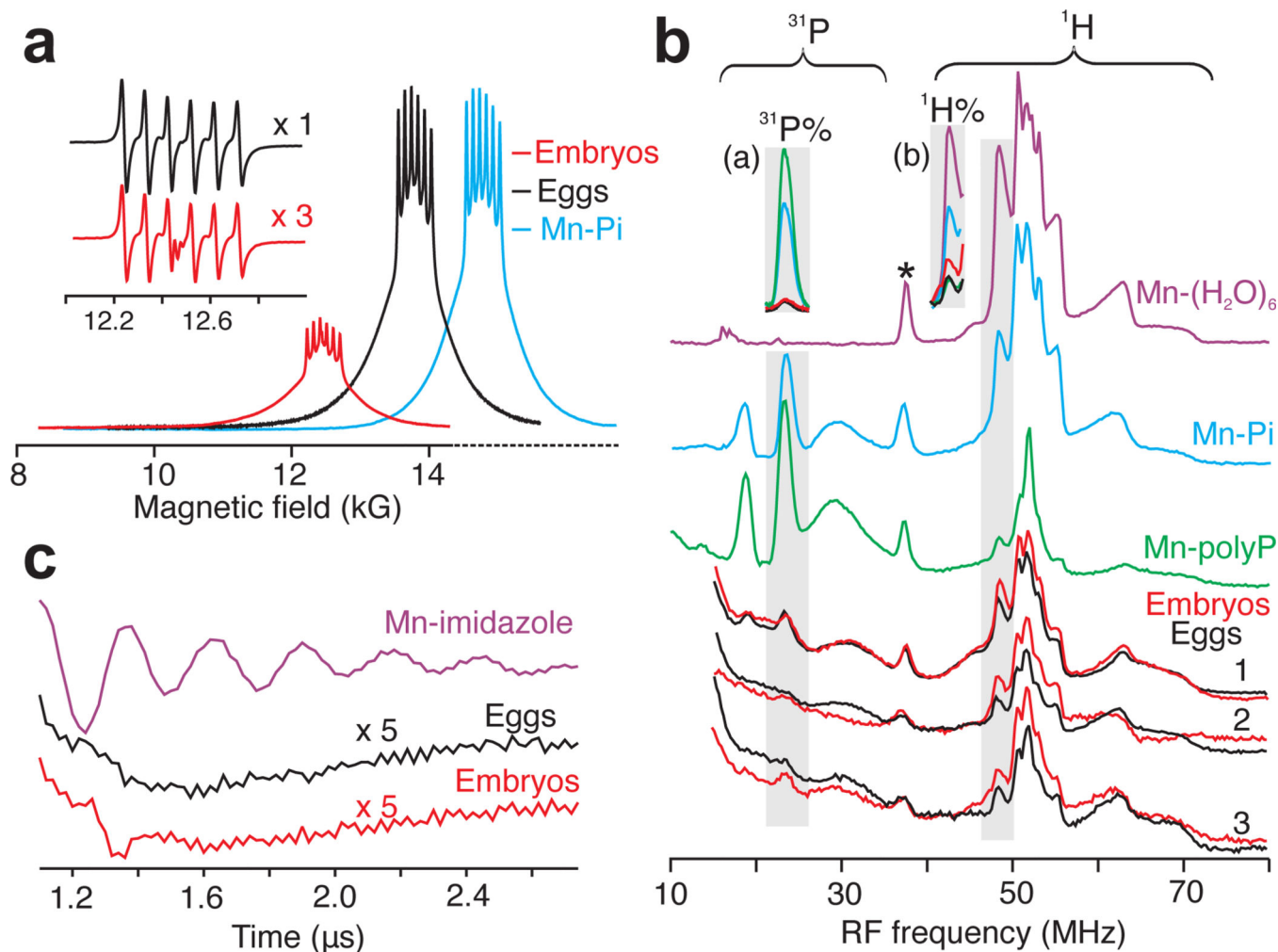
Figure 3: Paramagnetic resonance measurements of eggs and embryos.
EPR confirms a post-fertilization decrease in manganese, while EPR, ENDOR, and ESEEM demonstrate that the majority of intracellular manganese is bound to a low molecular weight carboxylate. 35-GHz, 2K EPR/ENDOR/ESEEM Spectra: **a.** Representative absorption-display CW-EPR spectra of Mn^{2+} in: frog eggs (black); embryos (red; 1/3 amplitude of eggs, ~30 ± 10% remaining, see text); Mn-orthophosphates Mn-Pi (blue; scaled to eggs); EPR spectra of frog eggs/Mn-Pi offset by +1,350/+2,350. *Inset:* digital derivatives of the spectra, accentuating the six-line ^{55}Mn hyperfine pattern around 12kG magnetic field. **b.** $^{31}P/^{1}H$ Davies pulsed-ENDOR spectra of: intact frog eggs and embryos; solution Mn^{2+} complexes (aquo [H_2O], orthophosphate [Pi], polyphosphate [polyP]). Braces represent frequency ranges of ^{31}P and ^{1}H ENDOR signals. ENDOR spectra are normalized to Mn^{2+} concentration; thus $^{31}P\%$, $^{1}H\%$ peaks (gray highlight) represent absolute ENDOR responses. *Insets:* (a) $^{31}P\%$ ENDOR responses of exemplars and eggs/embryos from frog 3; (b) corresponding $^{1}H\%$ ENDOR responses. (* is a ^{55}Mn ENDOR signal) **c.** ^{14}N 3-pulse ESEEM timewaves of: eggs (black); embryos (red), each multiplied by 5 for ease of comparison; solution Mn-imidazole complex (purple). *Experimental conditions:* see Supplemental Methods (**b, c**, Fields ~ 12.5k G). In **a.**, **b.**, and **c.** n = 3 biologically

independent replicates of intact frog eggs and embryos examined over 3 independent experiments.
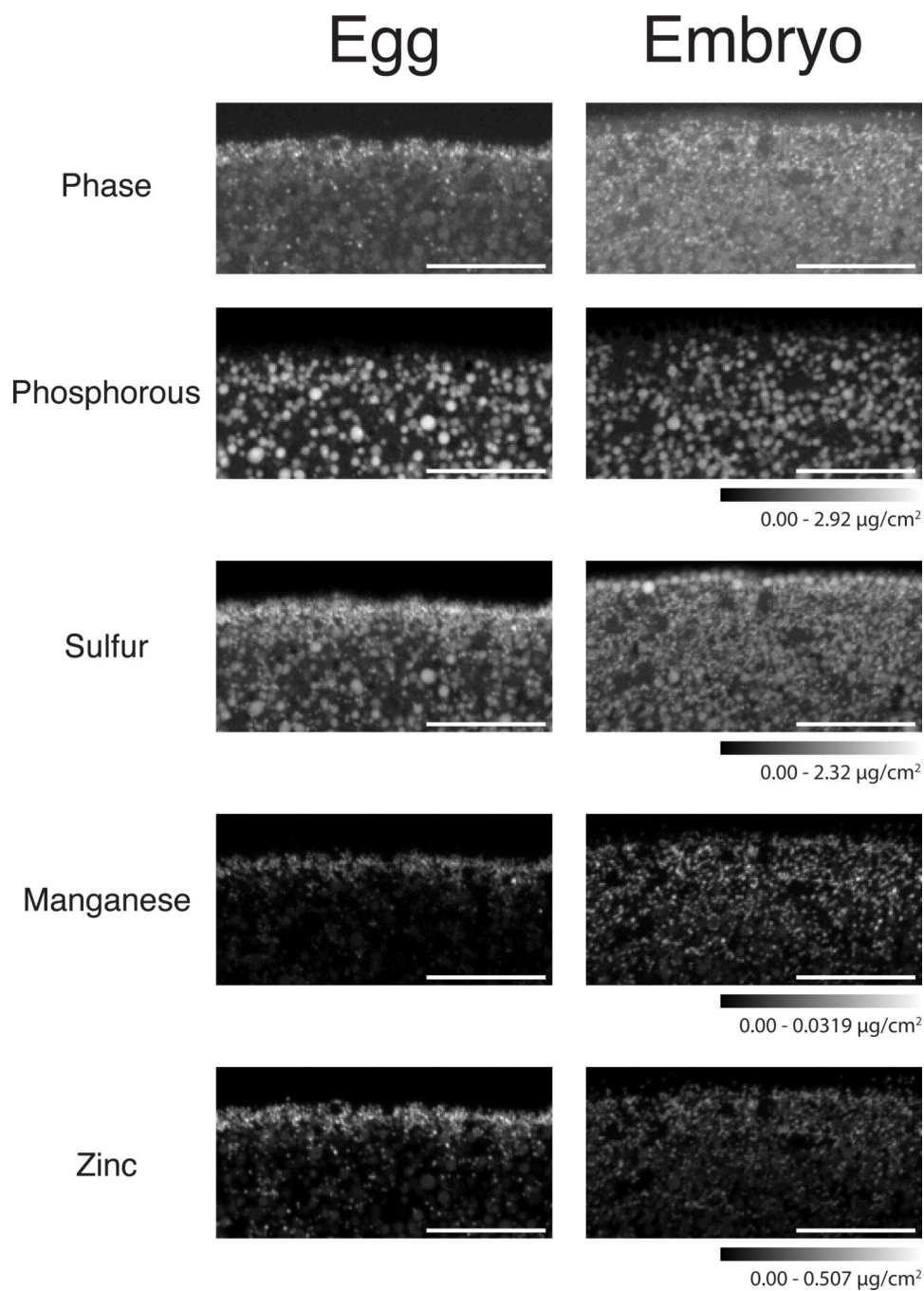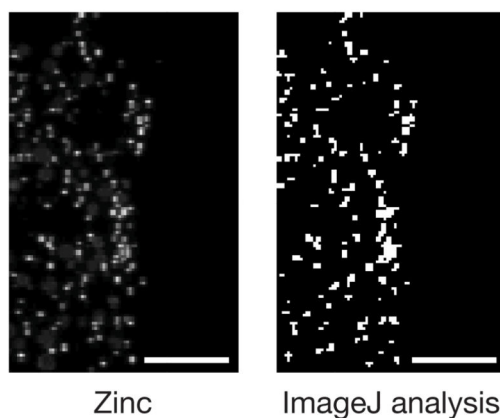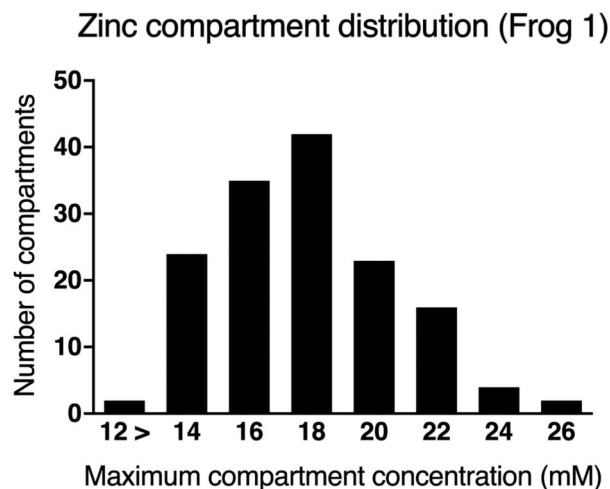
Figure 4: Synchrotron-based X-ray fluorescence mapping of egg and embryo cortices.
X-ray fluorescence microscopy images of the animal pole of fixed *Xenopus* egg/embryo slices show zinc and manganese are localized in small cortical compartments. Scale bar = 20 μm. Images acquired at Beamline 2-ID-D at Argonne National Laboratory. Pixel size: 300 × 300 nm, slice thickness: 2 μm, scan time: 500 ms/pixel. These are representative images of slices of eggs/embryos from 7 different frogs imaged over 3 experiments.

a

	Average µg/cm^2	Voxel Concentration (mM)
Mn	$4.0 \pm 1.1 \times 10^{-3}$	2 ± 0.5
Co	$5.9 \pm 6.7 \times 10^{-3}$	3 ± 3
Ni	$6.7 \pm 1.7 \times 10^{-4}$	0.3 ± 0.07
Cu	$2.4 \pm 1.5 \times 10^{-2}$	10 ± 6
Zn	$5.0 \pm 0.66 \times 10^{-2}$	20 ± 3

Mean ± SD. n = 3 frogs

b

Zinc   ImageJ analysis

c

Zinc compartment distribution (Frog 1)

Figure 5: Animal pole egg metal compartment contents.
Metals are stored at millimolar concentrations in the cortical compartments. **a.** Concentrations of metal compartments in the egg animal pole. Voxel size $300 \times 300 \times 400$ nm. Data acquired at Beamline 2-ID-D, Argonne National Laboratory. Values reported as mean ± SD, n = 3 slices of eggs analyzed from separate frogs examined over 2 independent experiments. **b.** Representative image of zinc distribution and thresholding of areas of high zinc concentration. Scale bar = 10 µm. Images acquired at Beamline 2-ID-D at Argonne National Laboratory. Pixel size: 300×300 nm, slice thickness: 400 nm, scan time: 500 ms/pixel. **c.** Representative plot of the zinc concentration distribution of small metal compartments found in a slice. n = 156 compartments.
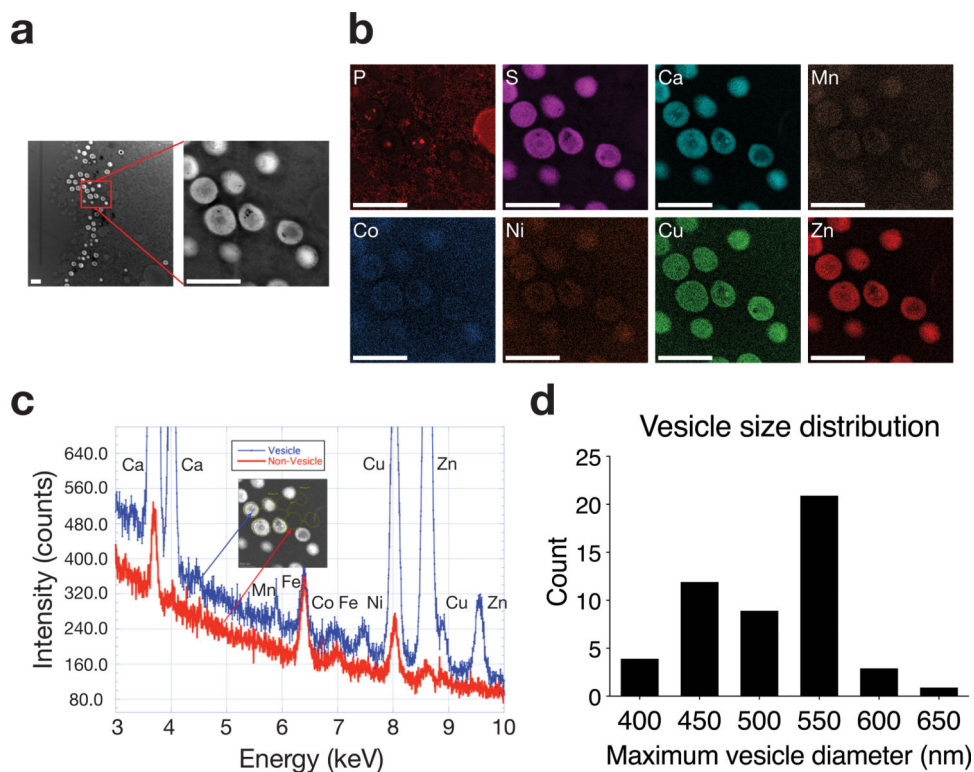
Figure 6: Analytical electron microscopic analysis of egg animal pole cortical vesicles.
AEM demonstrates that multiple metals are stored in sub-micrometer vesicles in the animal pole. **a.** High Angle Annular Dark Field Scanning Transmission Electron Microscopy (HAADF-STEM) images of the cortex of the animal pole of a fixed *Xenopus* egg. **b.** Hyperspectral elemental intensity distribution maps: background corrected, with a 0.7-pixel gaussian blur, no normalization, no quantification. Same conditions as in **a**. Scale bar = 1 μm in both **a.** and **b**. These are representative images of 3 separate scans. **c.** Cumulative XEDS spectra confirming the presence of Ca, Mn, Co, Ni, Cu, and Zn in vesicles. These comparison spectra are obtained by summing individual spectra from an identical number of pixels in regions of interest which correspond to vesicle and neighboring non-vesicle areas. **d.** Histogram of vesicle diameter distribution from image in **a**. n = 50 vesicles measured.
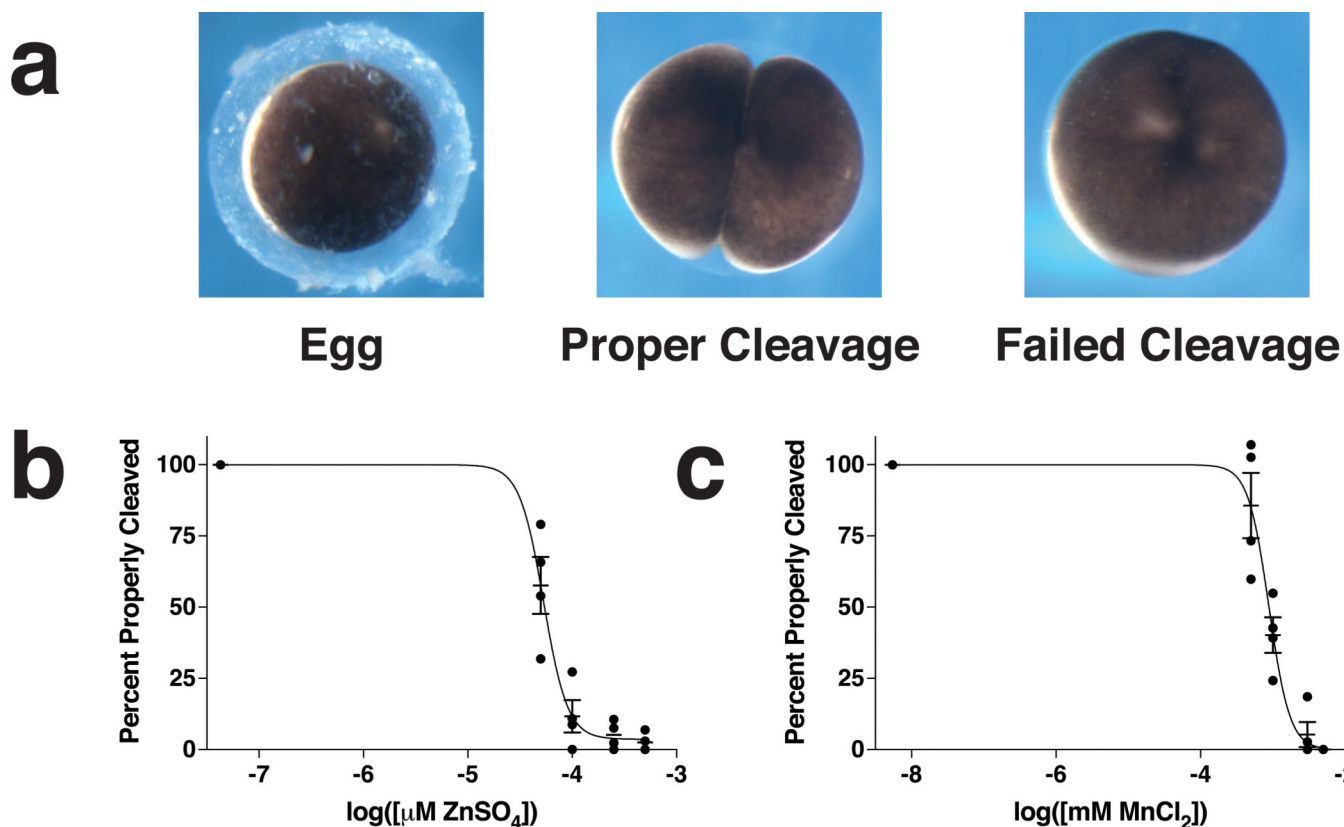
Figure 7: Extracellular zinc or manganese inhibits fertilization in a dose-dependent manner. Both extracellular zinc and manganese block fertilization. **a.** Images of an unfertilized *Xenopus* egg (in jelly coat), a properly dividing embryo (first cleavage), and an instance of failed cleavage (meiotic catastrophe). **b.** An IC_{50} graph of the effects of extracellular $ZnSO_4$ on the rate of fertilization. $IC_{50} = 53$ μM (95% CI = 47 – 61 μM, $R^2 = 0.94$). Data are presented as mean percentage of eggs properly fertilized ± SEM, n = 4 frogs (39 – 102 eggs per datapoint). See Supplemental Figure 11 for raw data. **c.** An IC_{50} graph of the effects of extracellular $MnCl_2$ on the rate of fertilization. $IC_{50} = 890$ μM (95% CI = 770 μM – 1.0 mM, $R^2 = 0.93$). Data are presented as mean percentage of eggs properly fertilized ± SEM, n = 4 frogs (36 – 100 eggs per datapoint). See Supplemental Figure 12 for raw data.